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14. ABSTRACT

Scleroderma is a disease where excess collagen is deposited in the skin and internal organs. The tissues become hard and in the end fail to function. To date there is no cure, nor is there an effective therapy that will control the deposition of the collagen. The goals of this application were to investigate the cellular signaling within fibroblasts that were mediated by the glycation end product, 3DG. We find that 3DG decreases the expression of collagens and therefore we proposed to understand the cellular signaling in fibroblasts in response to this compound. 3DG-collagen induced ER stress leading to caspase 3 activation, and decreased cell proliferation. Furthermore, 3DG-collagen induced changes in integrins and growth factor expression. Furthermore, we found that p38 MAPK was a crucial protein that regulated whether the fibroblast responded to a stress signal or a growth signal. The data obtained from this grant has allowed us to identify two candidate chemicals that lower collagen expression by SSc fibroblasts.

15. SUBJECT TERMS
3-deoxyglucosone (3DG), scleroderma (SSc), caspase, collagen, integrin, extracellular matrix, fibroblasts ER stress, p38 MAPK

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INTRODUCTION

The goals of this application were to investigate the cellular signaling within fibroblasts that are mediated by the glycation end product 3-deoxyglucosone (3DG). 3DG is a byproduct of glucose metabolism and can be found in high levels in the serum of patients with diabetes and contributes to diabetic complications such as chronic wounds. In the case of diabetics, they do not produce enough collagen to close the wound and we believe that 3DG contributes to this pathology. In the light of this, we investigated if 3DG was also able to down regulate collagen in the fibrotic disease, scleroderma (SSc). In the preliminary data in the grant, we demonstrated that collagen expression and TGF-β were decreased in fibroblasts cultured on 3DG-modified collagen matrices. Therefore, we stated in the abstract the following; this grant seeks to better understand the altered signaling between the ECM and fibroblasts isolated from the fibrotic lesions from SSc patients. The goals of the experiments proposed are a natural progression of the provocative preliminary data and will investigate the mechanism as to how 3DG can modify the signaling from the ECM via integrins, signaling through the ERK pathway. **Specifically, we propose that fibroblasts respond differentially to ECM that has been modified by 3DG. We hypothesize that this modification causes a feedback signal through the ERK pathway into the fibroblasts that results in the altered expression of pertinent transcription factors that in turn affect COL1A1, COL3A1, elastin, fibrillin-1, CTGF, and TGF-β gene expression.** More importantly, we believe that this mechanism can be utilized to modulate the fibrotic events observed in SSc.

BODY

The goal of this application was to understand the signaling pathways that were involved in the observed decreased collagen expression by scleroderma (SSc) fibroblasts when cultured on 3DG-collagen and to determine if these pathways could be used to downregulate collagen expression when not using 3DG-collagen. Our initial studies were to further elucidate the basic signaling mechanism of 3DG-collagen.

We employed pairs cell lines from the affected/involved region of the dermis or unaffected/uninvolved region of the dermis from patients with SSc. These paired cell lines were taken from affected and unaffected skin from scleroderma patients. Affected skin is where these is a clinically identified fibrotic lesion and the unaffected skin comprises of a non-fibrotic/clinically normal areas; however, although this latter area is considered to be clinically normal it is not normal in regards to gene expression when compared to control fibroblasts (individuals without fibrosis). Cells from Patient #4 (affected and unaffected fibroblasts) grew poorly in culture and these cells were discarded. We also obtained 7 aged matched normal cell lines from the Coriell Institute (Camden NJ). Based on the data below, we determined that it is important to have aged matched controls in these experiments as collagen expression decreases with increasing age (Fig. 1).

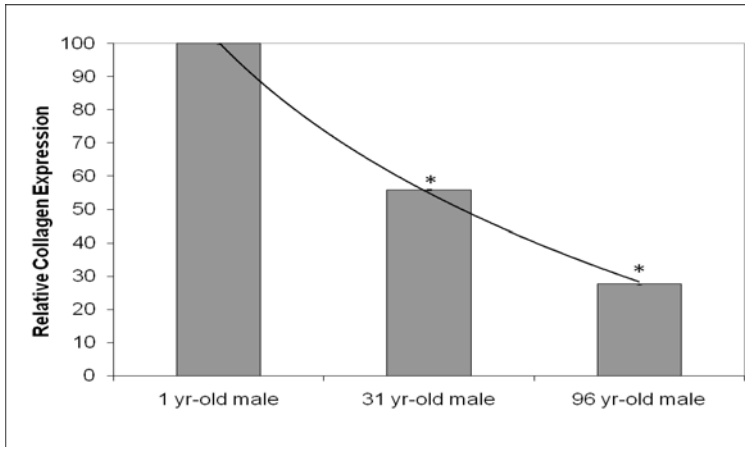

Figure 1. Collagen COL1A1) expression declines with chronological aging. Fibroblasts from the three cell lines were cultured (passage 2-4), RNA extracted, converted to cDNA and assayed for COL1A1 transcripts and normalized to β-actin expression. In this experiment, we assumed that the 1 yr-old had 100% collagen expression and expression for the 31-yr old and 96-yr old were corrected for relative expression to the 1-yr old. These differences were found to be statistically significant **(*P<0.0001)**

Preparation of the Collagen Lattices: Human collagen solution was purchased from Stem Cell Technologies and diluted in PBS to a concentration of 0.067 mg/ml according to Kessler et al (1). The culture dishes were flooded with collagen and incubated for 2 h at 37^{o}C. Dishes were then gently washed three times with sterile PBS and either used immediately or the collagen was further modified with 3DG overnight. Unincorporated 3DG was removed from the plates by gently washing the matrix three times with 5 ml PBS prior to plating with fibroblasts.

We determined that fibroblasts isolated from older individuals was more sensitive to the 3DG-modified collagen matrices and expressed

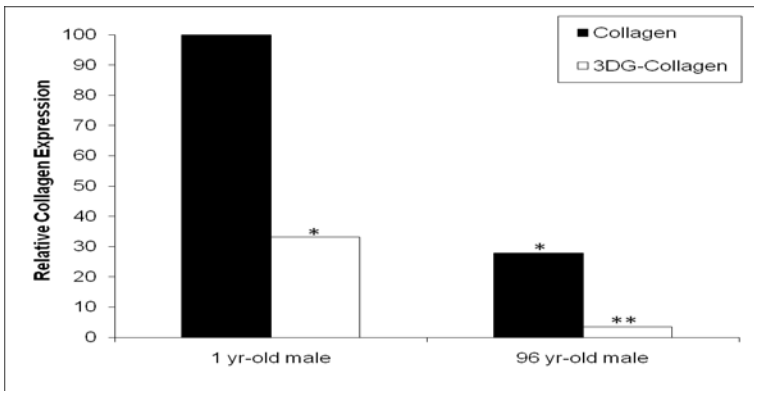

Figure 2. Fibroblasts from older individuals are more sensitive to 3DG-collagen. 100ng of cDNA was assayed for COL1A1 transcripts and normalized to β-actin. We found the 1 yr-old had 33% of the expression of collagen when fibroblasts were cultured on 3DG-modified matrices compared to the same fibroblasts cultured on non-modified matrices. The 96 yr-old had 12% of the expected collagen expression when cultured on 3DG-modified collagen matrices and compared to the same fibroblasts cultured on non-modified collagen matrices. When we compared COL1A1 expression of the 96 yr-old fibroblasts cultured on 3DG-collagen to the 1 yr-old fibroblasts culture on c, we found that the 96 yr-old expressed 3.4% of the expected COL1A1 transcripts. These differences were found to be statistically significant **(*P = 0.001; **P<0.0001).**

significantly less collagen than did fibroblasts from younger individuals (Fig. 2). The expression of collagen in control fibroblasts cultured on normal collagen was normalized to 100% and these fibroblasts when cultured on 3DG-collagen expressed approximately 1/3 of the starting amount. However, fibroblasts from the 96-yr old individual express of collagen was 28% of that expressed by the 1-yr old fibroblasts and this was found to decline to 12% in fibroblasts cultured on 3DG-collagen (Fig. 2). This indicates that fibroblasts from older individuals are more sensitive to alterations of the extracellular matrix.

We have an extensive panel of fibroblasts from individuals of all ages and we found that this difference in collagen expression with increasing chronological age was surprising. As all our SSc patient cell lines were obtained from females who were between 40-50 years of age, we have had to select corresponding normal cells from females in that age range. We also had to be strict in our matching criteria for passage number (cell doublings) of the fibroblast cell lines and their comparison to normal lines with the same passage number (cell doublings). For example, when we compared the expression of collagen type I and III in the same cell line at passage 6 and 16, we found that with increasing cell doublings, collagen expression is decreased (Fig. 3). This data is unpublished.

Therefore, due to the results of these extensive initial studies, it was demonstrated that for the collagen transcripts that we were investigating and the corresponding effects of 3DG on fibroblasts, it is very important that we carefully match the cell lines from the same age and same passage number.

All SSc cell lines received from Dr Feghali-Bostwick (University of Pittsburgh) were re-established in the laboratory and determined to be fibrotic when compared to the normal cell lines by the analysis of the expression of collagen genes (collagens type I and type III) by real time PCR quantification. All comparisons were corrected for β-actin expression. Our initial studies have focused on fibroblasts from the active lesions of these patients.

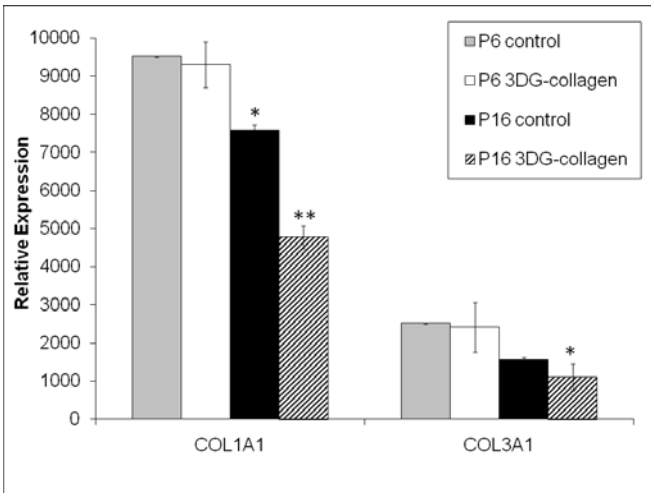

Figure 3. Increased passage number (cell doublings sensitizes fibroblasts to 3DG-collagen. 100ng of cDNA was assayed for COL1A1 and COL3A1 transcripts and normalized to β-actin. Statistical analyses were determined between passage 6 cultured on non-cross-linked matrices and was found to be statistically significant **(*P = 0.01; **P <0.001).**

Type I collagen expression in affected and unaffected cell lines from patients with SSc. It is known that there can be variability in collagen expression in fibroblasts established from biopsies from patients with SSc. This is due to variability in disease conditions and the site at which the biopsy sample was taken. Therefore we determined the collagen expression in primary fibroblasts from affected and unaffected skin explants at passage 2-4. It has further been reported that the increased collagen expression in SSc fibroblasts declines with increasing passage number and this is due to either apoptosis of myofibroblasts in the cell line, or de-

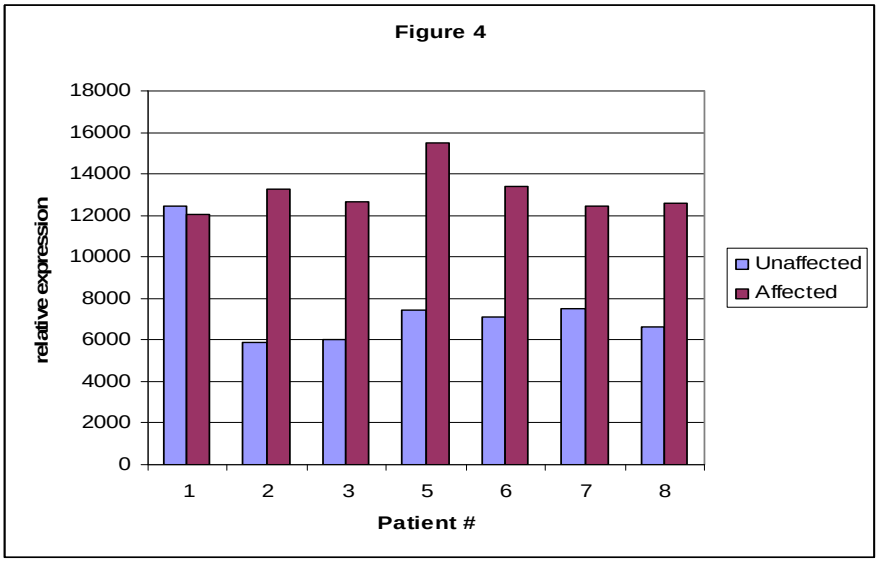

Figure 4. COL1A1 expression in the affected and unaffected fibroblast cell lines from patients with SSc. Fibroblast lines received from Dr Feghali-Bostwick were re-established in the laboratory and grown to confluence in a large flask. The lines were split to maintain fibroblast growth and a proportion of the fibroblasts were assayed for COL1A1 expression. All collagen transcripts were normalized to β-actin expression. Unaffected values are represented in light blue and the affected cell lines are represented in purple. As anticipated, with the exception of one affected line, all fibroblasts from the affected skin expressed more COL1A1 **(P = 0.001)**.

differentiation of myofibroblasts into quiescent fibroblasts. Therefore, SSc cell lines were periodically tested to determine collagen expression. Those lines that were deemed to be no longer fibrotic were discarded from the study and new lines were requested from our collaborator in Pittsburgh.

When we study the cell lines that we received, we found that patient #1 in both the unaffected and affected cell lines had similar levels in type I collagen. This suggests that the unaffected cell line from this individual was not unaffected and the biopsy may have been on the edge of the affected area (Fig. 4).

We found that by simply culturing SSc fibroblasts with 3DG we were able to lower collagen mRNA levels (Fig. 5) and that this effect was greater than culturing these cells on 3DG-collagen (not shown). Therefore we performed our experiments with 3DG added. Furthermore, when fructoselysine is metabolized, 3DG is liberated by the cell and we confirmed that collagen expression could also be reduced by fructoselysine (Fig. 5). In addition to lowering collagen mRNA, we observed that fructoselysine had a greater effect that 3DG. We believe that manufactured 3DG has degraded and that 3DG produced by fructoselysine is biologically fresh and more reactive (Fig 5).

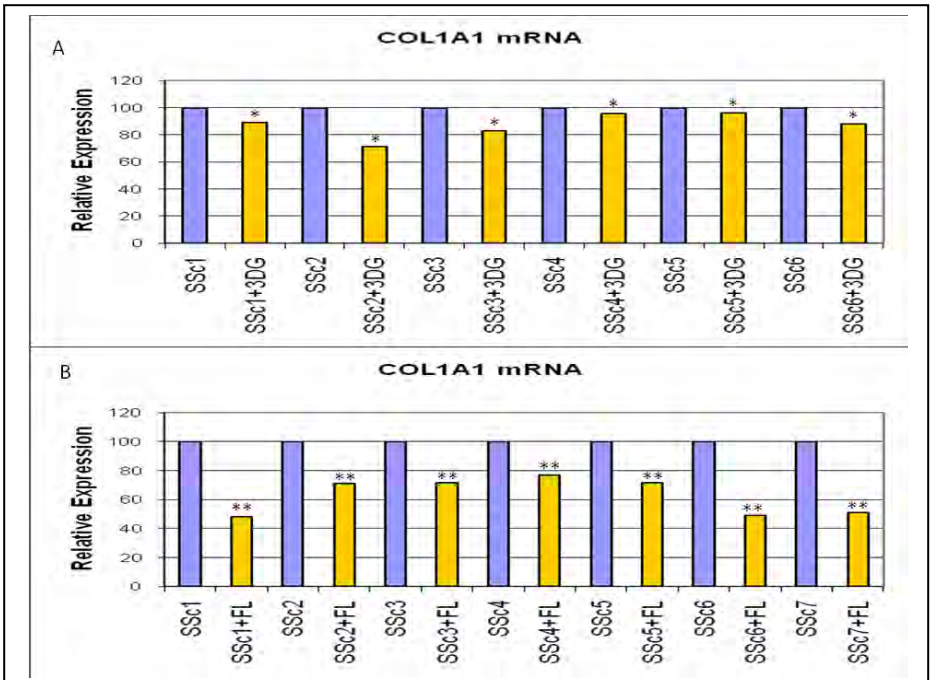

Figure 5. 3DG and fructoselysine lower collagen expression in SSc Fibroblasts. SSc fibroblasts were treated with and without 1 mM 3DG (Panel A) or 5 mM fructoselysine (Panel B). Type I collagen (COL1A1) transcripts were measured and normalized to β-actin **(*p=0.019, **P=0.0002)**.

Not all advanced glycation end products decrease collagen expression in fibroblasts. Previous publications have suggested that methylglyoxal (MG) induces collagen expression in fibroblasts and therefore we investigated this precursor in our model (Fig. 6). We found this effect to be intriguing and further analyzed MG-collagen in association with 3DG-collagen.

3DG and MG modify collagen matrices differently. The α-dicarbonyl compounds 3DG and MG are reactive compounds that are capable of reacting inter-molecularly and intra-molecularly with amino groups resulting in the formation of stable AGEs. Electron microscopy analysis revealed that glycated collagen fibers had larger, irregular diameters (2) caused by the expansion of intermolecular spaces

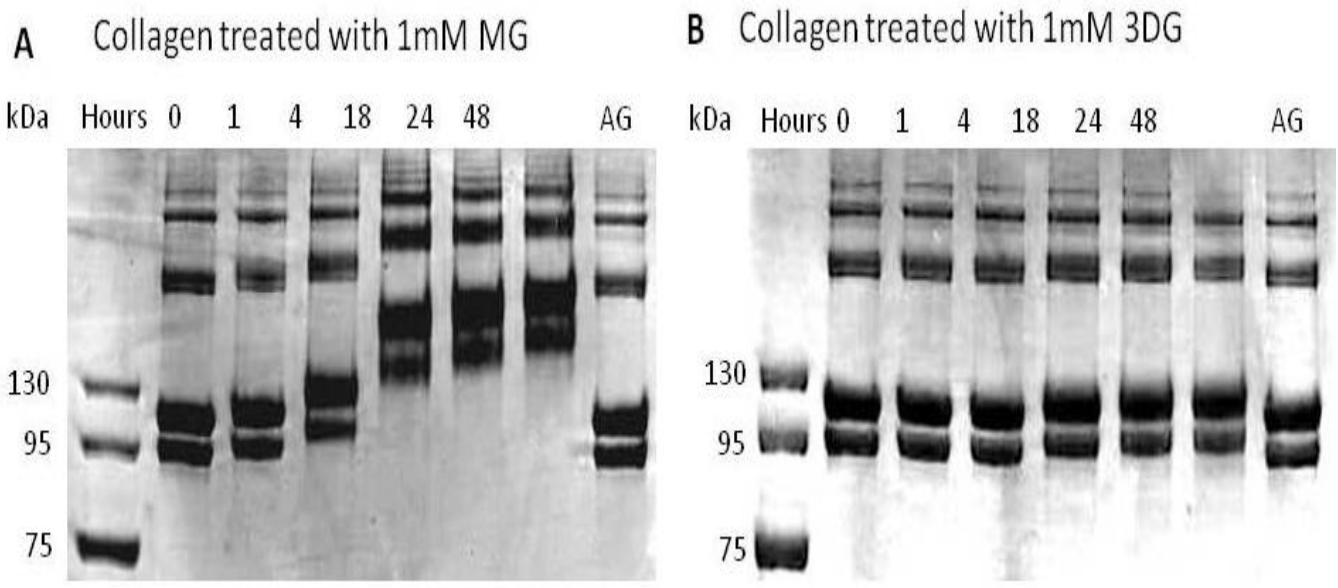

Figure 6. Rate of modification of collagen by 3DG and MG. Normal human collagen was incubated for either 1mM MG (Panel A) or 1mM 3DG (Panel B) at 37°C for increasing amounts of time: 1, 4, 18, 24, and 48 h. In addition, we incubated collagen with the AGE precursor for 48 h supplemented with 5mM aminoguanidine (AG). The protein was size fractionated on a 10% SDS denaturing gel and stained with Coomassie for 60 min and destained prior to scanning.

between the collagen fibrils (3). Therefore, we incubated collagen with 1mM 3DG or 1mM MG for increasing amounts of time to determine the rate of modification of the collagen molecule (Fig. 6). Collagen was then size fractionated the protein on gels and stained with Coomassie to determine if these AGE precursors affected the collagen fibrils. We found that MG treated collagen at 1 h had a size shift, with an increasing shift in apparent molecular weight with increasing time. In contrast, we saw no detectable shift in the apparent molecular weight with 3DG until 18 h and with increasing time, the shift in apparent molecular weight of the collagen did not increase beyond that which was observed at 24 h. To confirm the specificity of the AGE precursor to collagen modification, we incubated the AGE precursor with 5mM aminoguanidine (AG) for 48 h and this completely inhibited the shift in size fractionation of the collagen. This confirms that the shift in apparent molecular weight of collagen was due to the AGE precursor. As 3DG exhibited a slower rate of collagen modification, we also treated collagen with 1mM 3DG for 6 days and observed no further size shift in collagen than that which was observed at 48 h (data not shown). This data suggests that the modification of collagen by 3DG is different from the modification modulated by MG. This data was published in Matrix Biology, 2010.

Morpholinofructose (MF) induces 3DG in fibroblasts and concordantly decreases hydroxyproline (total collagen). Many types of cells are able to utilize MF, which is an alternative substrate for the enzyme fructosamine 3-kinase and results in increased levels of 3DG. We investigated the production of 3DG by confluent dermal fibroblasts cultured with MF. Two 70 mm dishes each from the 9 different fibroblast cells lines were cultured at low passage (passage 2–4), with or without 10 mM MF for 24 h. 3DG liberated into the media was extracted and measured by GC-MS. Fibroblasts cultured without MF liberated 780 $\pm$ 213 μmol/l of 3DG compared to 1893 $\pm$ 161 μmol/l with MF; ($p<0.0001$). Likewise, intracellular 3DG levels in fibroblasts were found to be elevated; 8.3 $\pm$ 3.0) μmol/l 3DG in the controls vs. 27.9 $\pm$ 6.7 μmol/l 3DG with MF, ($p<0.0001$). Five of the primary fibroblast cell lines cultured with MF were assessed for hydroxyproline (total collagen) and were found to express less collagen than the fibroblasts cultured without MF. Fibroblasts cultured with 10 mM MF had an average hydroxyproline expression of 63.6% of that expressed by the control ($p=0.0032$). The decrease in the expression of COL1A1 and COL3A1 was confirmed at the mRNA level. Fibroblasts cultured with MF also had a corresponding decrease in mRNA of COL1A1; 62%, $p= 0.0002$ and COL3A1; 39%, $p<0.0001$ compared to the control.

3DG and MG cross-linked collagen matrices modulate COL1A1 and COL3A1 expression differently. Collagen matrices were treated with 1 mM 3DG, 1 mM MG, or 10 mM MG overnight and six fibroblast cell lines were cultured on the matrices for 3 days. RNA was extracted and COL1A1 and COL3A1 mRNA

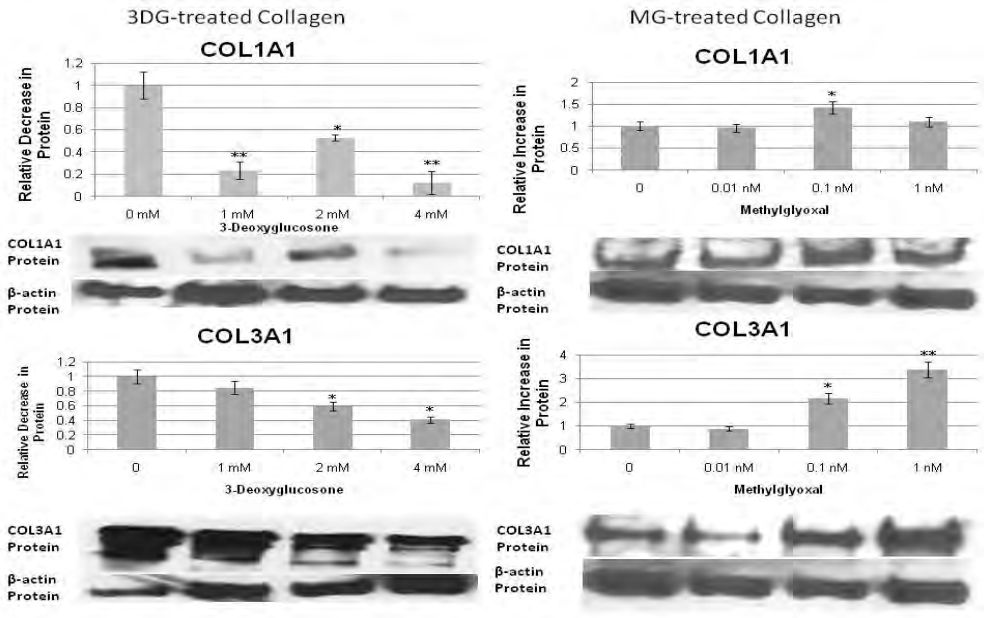

Figure 7. Expression of COL1A1 and COL3A1 mRNA transcripts in fibroblasts cultured on collagen matrices modified by 3DG or MG. Fibroblasts cultured on collagen matrices cross-linked with increasing amounts of 3DG (0, 1, 2 or 4 mM) or MG (0, 1, 2, and 5 mM) for 24 h were assayed for COL1A1 and COL3A1 protein. Bands sizes were quantified by ImageJ and plotted as a percentage of the normal collagen expression. Values are presented as mean +/- SD, ***P = 0.02; **P = 0.01.**

transcripts were quantified by SYBR green real time PCR. With the MG treated collagen matrices, fibroblasts increased the expression of COL1A1 and COL3A1 mRNA but this was most pronounced at 10 mM MG (not shown). When we titrated MG and measured COL1A1 and COL3A1 protein by Western blotting, we found that COL3A1 expression was more sensitive to the presence of a MG modification (Fig.7). In contrast, fibroblasts cultured on the 3DG collagen matrices consistently demonstrated a decrease in expression of COL1A1 and COL3A1 mRNA transcripts (COL1A1, p=0.008 and COL3A1, p=0.0005, not shown). The decrease in collagen expression (COL1A1 and COL3A1) with 3DG was confirmed at the protein level by Western blotting and COL1A1 appeared to be more sensitive to the 3DG modification (Fig.7) Both COL1A1 and COL3A1 had significant declines in protein with 1 mM 3DG. This data was published in Matrix Biology 2010.

Transforming growth factor-β1 and β1-integrin mRNA is down regulated in response to the 3DG-collagen but upregulated with MG-collagen. TGF-β1 is a critical cytokine involved in the basal regulation of COL1A1, COL3A1, and β1-integrin. Upregulation of TGF-β1 is observed in fibrotic disorders and results in a co-ordinate increase in the expression of the collagen genes. Therefore, we investigated the expression of TGF-β1 in fibroblasts, using the same RNA that was used to determine the COL1A1 and COL3A1 transcripts in Fig. 7. In keeping

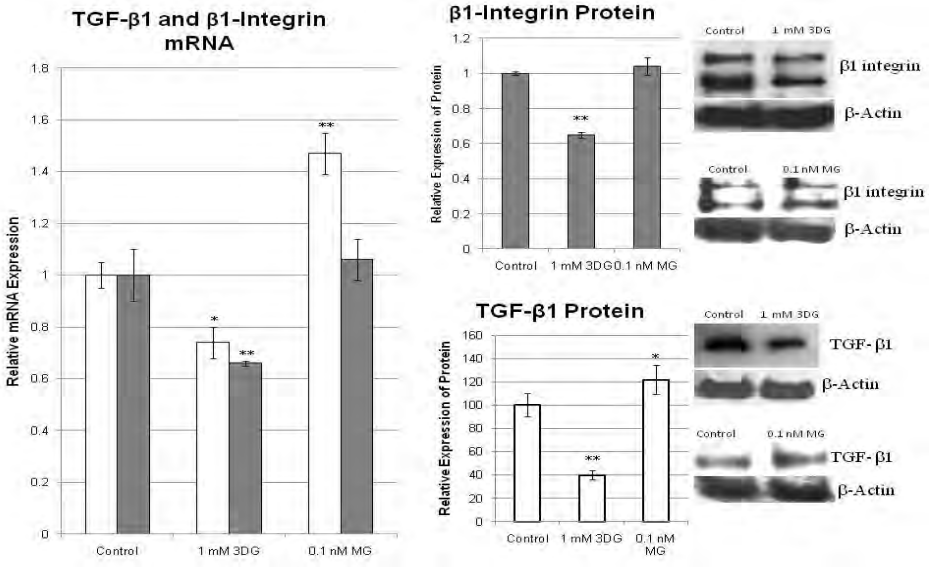

Figure 8. TGF-β and β1-integrin expression is decreased by 3DG modified collagen matrices and increased with MG modified collagen matrices. RNA extracted from six fibroblast cell lines in Figure 2 and assayed for TGF-β1 (white bars) and β1-integrin (gray bars). Values are presented as mean +/- SD. In concordance with the decreased collagen expression with 3DG and the increased collagen expression with MG, there was a decrease in TGF-β1 mRNA in cells cultured on 3DG modified collagen matrices and an increase in TGF-β1 expression in cells cultured on MG-modified collagen matrices. Protein analysis demonstrated that MG and 3DG concordantly increased or decreased β1-integrin and TGF-β1, respectively. Protein concentrations were corrected for β-actin protein. ***P < 0.03, **P < 0.001**.

with the decreased expression of the collagen genes, we found TGF-β1 to be decreased in response to 3DG-treated collagen matrices (Fig. 8; p=0.03). However, although we observed an increase the expression of TGF-β in response to MG treated collagen matrices, this was not statistically significant (Fig. 8). TGF-β1 protein was decreased in fibroblasts cultured on 3DG-treated collagen matrices (p=0.001; Fig. 8) and increased in MG-treated collagen matrices (p=0.001; Fig. 8). Furthermore, in analyzing β1-integrin we found that the mRNA was also decreased with in cells cultured on the 3DG treated matrices (p=0.0002) but increased in cells cultured on the MG treated matrices (p=0.026; Fig. 8). Western blot analysis confirmed that cells cultured with 3DG decreased β1-integrin protein (p=0.001l; Fig. 8) whereas MG increased β1-integrin protein in fibroblasts (not significant; Fig. 8). This data was published in Matrix Biology.

We found that there was a lot of interplay between Aims 1 and 2 and that signaling from integrins (Aim 2) played into the response of the SSc fibroblast to 3DG-collagen in Aim 1. Therefore many of the experiments were done concurrently as we identified the signaling pathways mediated by 3DG-collagen. We performed all the experiments proposed in Aims 1 and 2 but with greater complexity due to the signaling pathways that were involved.

Specific Aim 1: This aim is involved in identifying the signaling pathway within the fibroblasts that is responsible for the decrease in ECM expression in response to the 3DG modified collagen matrices. We believe that the ERK pathway is involved in the response of fibroblasts to the 3DG modified collagen matrices.

A) Assessment of phosphorylated proteins in the ERK pathway. Specifically, we will perform a thorough investigation of the ERK signaling pathway by Western blotting for ERK1, ERK 2, MEK1, and MEK2 to determine if there is any alteration in the phosphorylation of these proteins. We will be analyzing 6 fibroblast cell lines isolated from SSc patients and 6 fibroblast cell lines from normal individuals. We already have these lines selected and available and we have used some of these lines in the preliminary data. We estimate that it will take 6 months for this initial analysis and to grow the fibroblasts and to repeat the experiment twice. We will also investigate the total expression of ERK1, ERK 2, MEK1, and MEK2 with immunofluorescence microscopy and flow cytometry. These analyses will confirm the Western blotting.

3DG-Collagen Decreases Fibroblast ERK1/2 and MEK1/2 Phosphorylation. Phosphorylation of ERK1/2 (also called p42/p44 MAP kinase) is important in intracellular signaling and plays a crucial role in promoting cell proliferation and differentiation (4-6). The ERK proteins are found at sites in the signaling cascades where the signals converge and activate ERK1/2. These signaling cascades can regulate cytoskeletal remodeling, cell migration, and promote cell cycle progression (7-9). We found that normal fibroblasts cultured on 3DG-

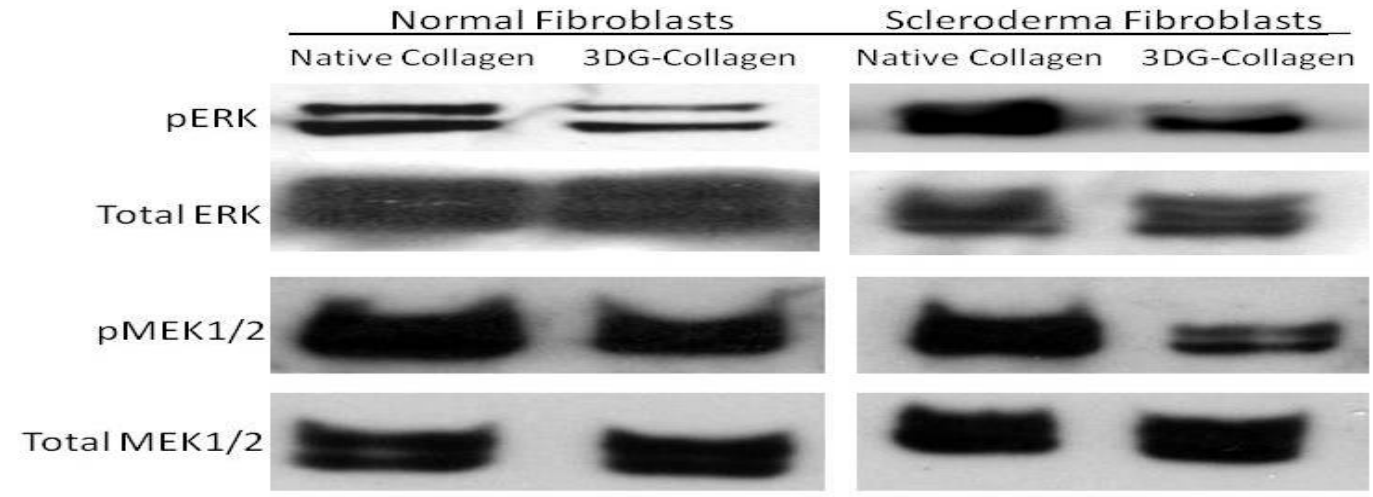

Figure 9. 3DG-collagen reduces pERK1/2 and pMEK1/2 signaling. Normal and SSc fibroblasts were cultured on native or 3DG-collagen for 72 h. Protein was extracted from the fibroblasts and size fractionated on polyacrylamide gels, transferred to PVDF membrane and probed for ERK1/2 using a specific antibody against phosphorylated ERK1/2. The densities of the bands were quantified by ImageJ and corrected for total ERK protein. Both normal and SSc fibroblasts decreased ERK1/2 phosphorylation when cultured on 3DG-collagen matrices; normal fibroblasts to 55% ± 2.12% **(P < 0.007)** and SSc fibroblasts to 63.6% ± 3.9% **(P= 0.0064)**. SSc fibroblasts significantly decreased MEK1/2 phosphorylation when cultured on 3DG-collagen matrices to 61.9% ± 2.1% of native collagen **(P= 0.005).**

collagen had ERK1/2 phosphorylation decreased to 55% ± 2.12% ($p<0.007$) compared to normal fibroblasts cultured on native collagen (Fig. 9). Likewise with the SSc fibroblasts, we found that there was a significant reduction in the phosphorylation of ERK1/2. ERK1/2 phosphorylation was decreased to 63.6% ± 3.9% of SSc fibroblast values cultured on native collagen ($p=0.0064$, Fig. 9). As ERK1/2 signals from MEK1/2, we wanted to determine if there was also an alteration in the phosphorylation of MEK1/2. After the bands were corrected for total MEK1/2 protein, we found that the averaged MEK1/2 phosphorylation was decreased to 61.9% when SSc fibroblasts. We found also that normal fibroblasts also had reduced MEK1/2 phosphorylation but 3DG-collagen did not decrease the phosphorylation of MEK1/2 significantly and it was found to be 90% of that observed on native collagen. The differences with SSc fibroblasts on 3DG-collagen was found to be statistically significant ($p=0.005$; Fig. 9).

The expression of phospho-ERK1/2 and phospho-MEK1/2 was confirmed by immunofluorescence in SSc fibroblasts and we observed the decreased staining of these proteins in cells when cultured on 3DG-collagen (Fig. 10).

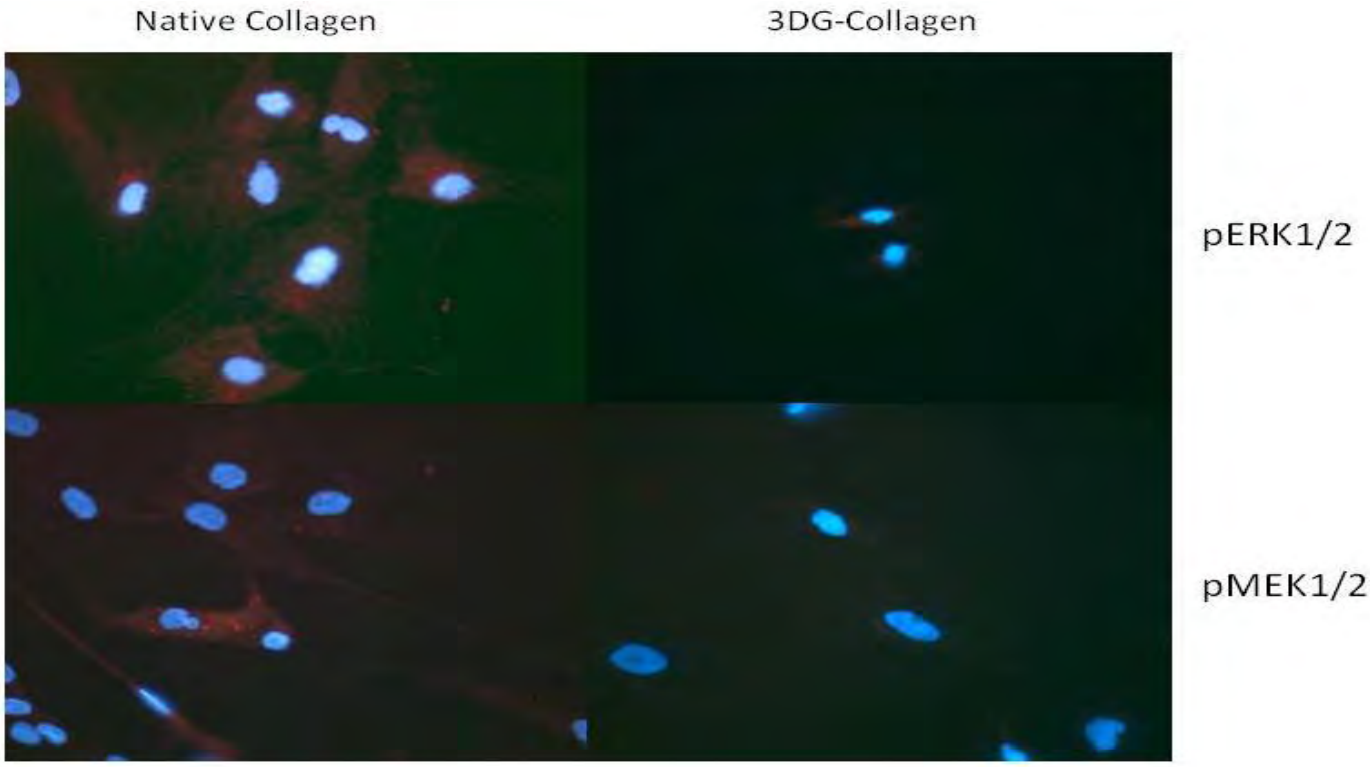

Figure 10. Histological analyses of ERK1/2 and MEK1/2 in fibroblasts cultured on native and 3DG-collagen. Fibroblasts were cultured on native or 3DG collagen and stained for ERK1/2 or MEK1/2, counterstained with DaPI to denote the nuclei (blue) and photographed. Note the intense staining of both ERK1/2 and MEK1/2 on native collagen, whereas there was very little staining for both ERK1/2 and MEK1/2 on the 3DG-collagen.

3DG decreases proliferation. The proliferation of fibroblasts on polypropylene in response to 2 mM 3DG, 1 mM MG, 2 mM MF and 40 mM Dyn15 were analyzed. One hundred fibroblasts were seeded in triplicate into 96-well polypropylene culture plates and cultured in 100 µl DMEM. After 24 h, the supplements were added and the cells cultured for an additional 24 and 48 h. Proliferation of the cells was measured according to the instructions from the Colorimetric (MTT) Assay for Cell Survival and Proliferation (Chemicon International). Proliferation at 0 h was determined by adding the supplements and then immediately adding the MTT reagent. Fibroblast proliferation on 3DG and MG cross-linked matrices was also measured. One hundred microliters of the diluted collagen was aliquoted into each well and incubated, cross-linked and washed as described above, then 100 fibroblasts were seeded into each well and the supplements were added after 24 h. Proliferation was measured at 0, 24 and 48 h. These experiments were repeated at least 5 times.

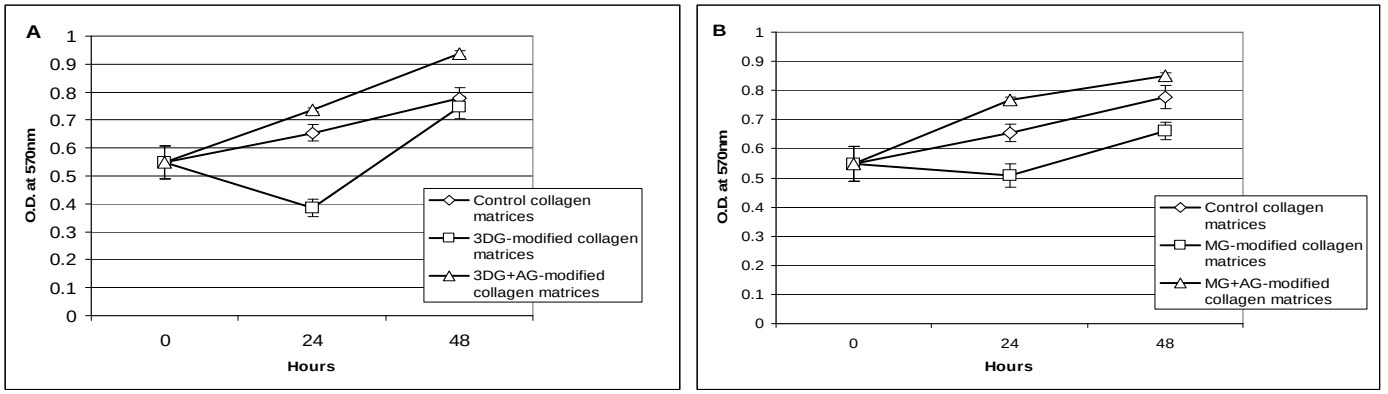

Figure 11A-B. Proliferation of fibroblasts on 3DG or MG cross-linked collagen matrices. The wells in a 96 well plate was coated with collagen and cross-linked with either 1 mM 3DG (Panel A) or 10 mM MG (Panel B) overnight. The wells were washed with three changes of PBS and then 200 fibroblasts were seeded onto the collagen and allowed to adhere overnight before the zero reading was made. The proliferation of the fibroblasts was measured at 0, 24 and 48 h, employing the MTT Cell Growth kit (Chemicon International). Panel A: proliferation of fibroblasts on 3DG modified collagen matrices and on 3DG + AG modified collagen matrices; Panel B: proliferation of fibroblasts on MG-modified collagen matrices and on MG + AG-modified collagen matrices. Differences were not found to be statistically significant at 48 h.

We investigated the viability of fibroblasts when cultured with 3DG and found that this compound induced cell death. The proliferation of normal fibroblasts was found to be altered when cultured with MF, 3DG, and Dyn15 (Fig. 11). Fibroblast cell lines were plated in triplicate and treated with the supplements for 0, 24 and 48 h. Cells treated with 1mM 3DG and 1mM MG did not proliferate during the 48 h and 3DG had a more adverse effect on fibroblasts than did MG (Fig. 11A). 2mM MF had a delayed effect on the fibroblasts and induced no cell proliferation in the first 24 h. As the cells metabolized the MF and produced more 3DG, the cells died resulting in approximately 20% of the starting O.D ($p<0.001$). Dyn15/meglumine that inhibits the metabolism of MF by blocking fructosamine 3-kinase activity allowed the cells to recover their proliferative capacity (Fig. 11).

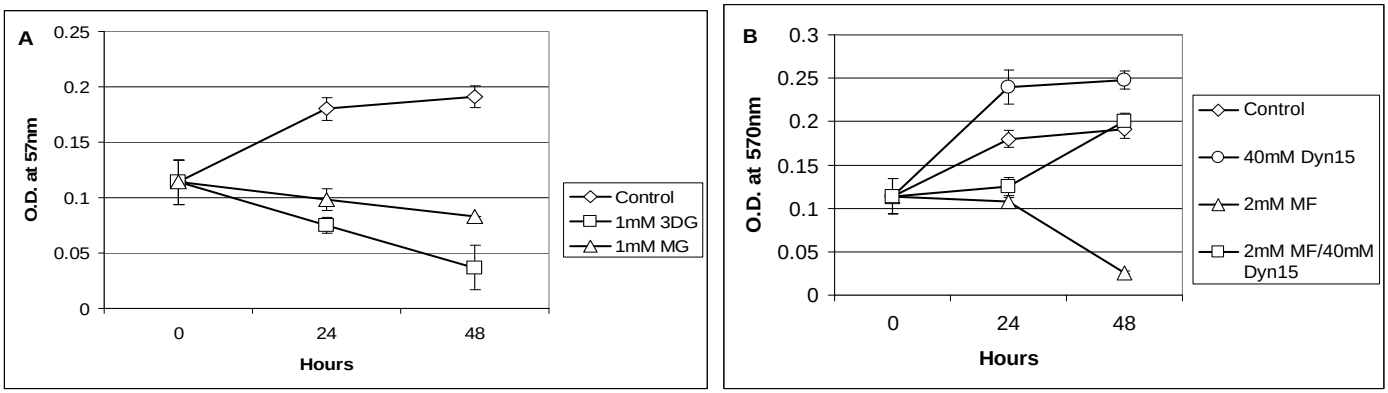

Figure 12A-B. Proliferation of fibroblasts cultured with of MF, MG, and 3DG. These experiments were performed on four separate occasions. Two hundred fibroblasts were seeded into a 96 well plate and allowed to adhere overnight before the zero hours was measured and the supplements added. The proliferation of the fibroblasts was measured at 0, 24, and 48 h, employing the MTT Cell Growth kit (Chemicon International). Panel A: proliferation of fibroblasts with 1 mM 3DG or 1 mM MG; Panel B: proliferation of fibroblasts with 2 mM MF or 2 mM MF and 40 mM Dyn15, or Dyn15. Differences were found to be statistically significant at 48 h comparing control vs. supplement:
*** P < 0.001**.

The cross-linking of collagen with 3DG or MG slowed the proliferation of fibroblasts on the matrices (Fig. 12), but the changes in proliferation of the cell was not altered as significantly as the fibroblasts which had 3DG or MG applied directly to the cells. Inactivation of MG and 3DG by AG recovered cell proliferation.

3DG induces caspase-3 activity. As the ERK pathway is involved in cell survival, we investigated the induction of apoptosis by the 3DG-modified collagen matrices. If we find that the 3DG-modified matrices affect cell viability, we will also investigate the phosphorylation of Akt and subsequent downstream proteins

including caspase 9, IκBβ and the forkhead transcription factors. We will also perform more traditional analyses for the investigation of apoptosis such as the TUNEL assay or *in situ* nick translation assays.

Eleven caspases have been identified in humans and they fall into two major categories: initiator and effector caspases and they can either be inflammatory or apoptotic. In the apoptotic caspase group, initiator caspases activate effector caspases by cleaving their inactive forms. Therefore, we investigated the effector caspase, caspase-3 to determine if there was any alteration in activity. Caspase 3 protein activity was measured as a marker of apoptosis activity using the Caspase-3 Colorimetric Correlate Assay according to the manufacturer's protocol (Assay Designs, Ann Arbor MI). AGE precursors have been implicated in inducing apoptosis and therefore we studied the role of 3DG in the induction of caspase-3 activity. Cells were lysed and caspase-3 activity was measured using the Caspase-3 Colorimetric Correlate Assay (Assay Designs) in fibroblasts cultured overnight with 1 mM 3DG, 2 mM MF (a substrate that the cells can utilize to make 3DG), and 40 mM Dyn15. Dyn15 is a compound that inhibits the metabolism of MF to 3DG. All assays were normalized to 100% activity of the control sample and each assay was performed in triplicate. We found that Dyn15 did not induce the activity of caspase-3, however, 3DG, and MF did (490% and 460%, respectively, Fig. 13). This was statistically significant; p=0.0004 for 3DG, and p=0.0004 for MF when compared to the control. Aminoguanidine chelates 3DG rendering it inactive. We observed that the inactivation of 3DG with 10 mM AG yielded normal activity of caspase-3 confirming that 3DG directly induces caspase-3 activity. As Dyn15 inhibits fructosamine 3-kinase, cells cultured with MF also yielded normal caspase-3 activity levels and 40 mM Dyn15 did not induce the expression of caspase-3 above normal levels.

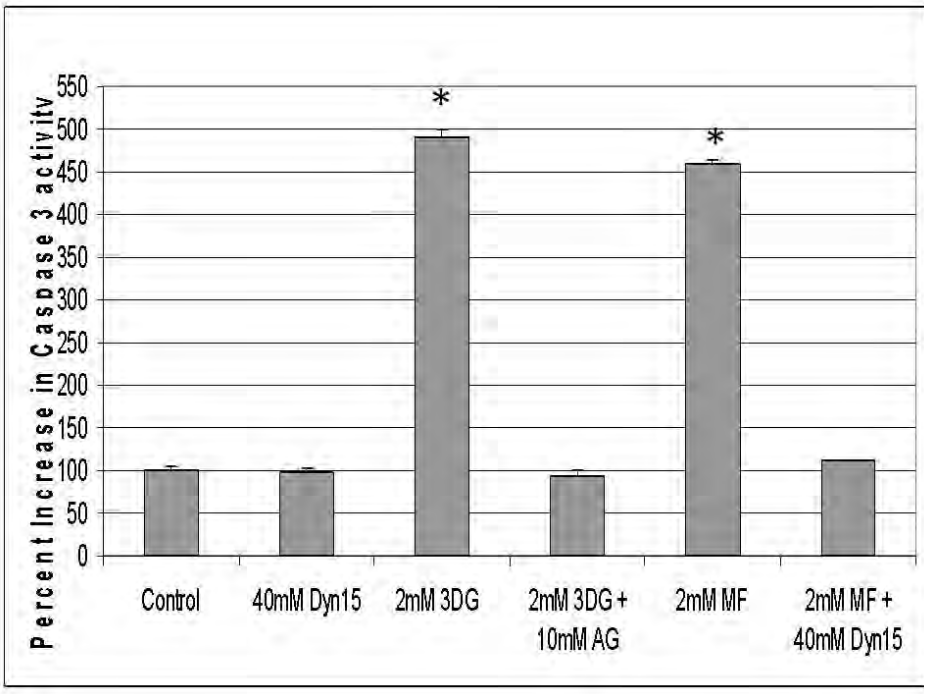

Figure 13. Expression of Caspase 3 activity in fibroblasts. Cells were cultured overnight with either 40mM Dyn15, an inhibitor of the enzyme fructosamine 3-kinase, 2mM 3DG, 2mM 3DG and 10mM aminoguanidine (AG), 2mM MF and 2mM MF+ 40mM Dyn15. Cells were lysed and caspase 3 activity was measured in the cell lysates according to the manufacturer's recommendations for the kit Caspase 3 Colorimetric Correlate Assay (Assay Designs) and compared to cells that remained untreated. The activation of the control cells was normalized to 100% and caspase 3 activation was determined by comparison to the normal values. We found that caspase 3 activity was increased by 3DG to 490% and MF to 460%. This was statistically significant; ***P = 0.0004.**

Both MG also induces Caspase-3 activation. AGE precursors have been implicated in apoptosis (10). Therefore, we also investigated if MG induced caspase-3 activation. Caspase-3 activation in cell lysates was measured in fibroblasts cultured overnight with 10 mM MG. MG induced a 4.9 fold increase in caspase-3 activity and this could be normalized with 5 mM AG (data not shown).

b) Transcription Factor Analysis. We will investigate alterations in the expression of 3 transcription factors that are responsible for the high level of collagen expression in SSc; Sp1, c-fos, and c-Myc. This thorough assessment will take approximately 8 months for completion. We will perform ELISAs on cell lysis from the 6 SSc and 6 normal fibroblast cell lines; Sp1, c-fos and c-Myc proteins to determine if there is any alteration in the total concentration of these proteins in the fibroblasts when cultured on 3DG modified matrices. In addition we will perform electrophoretic mobility shift assays on the promoter of COL1A1 (we already have these

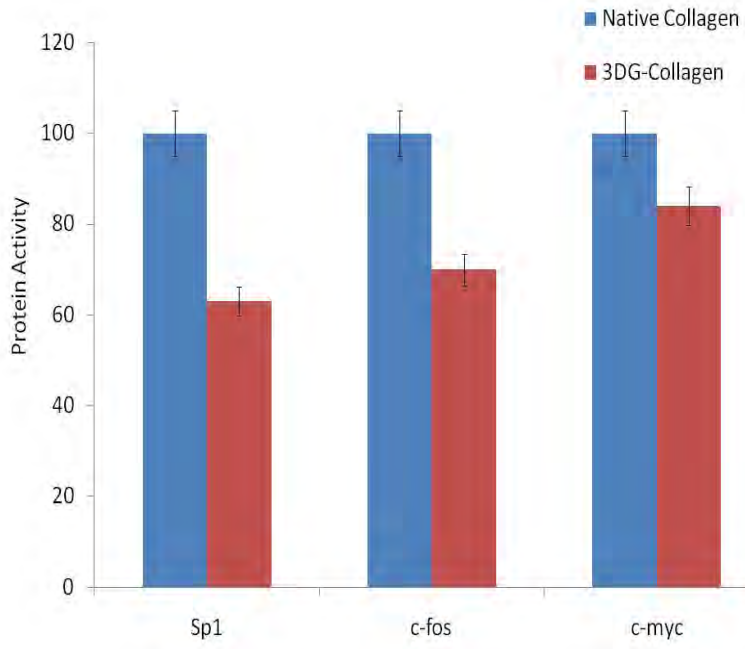

Figure 14. Transcription factor activity for Sp1, c-myc, and c-fos is decreased in fibroblasts cultured on 3DG-collagen. SSc fibroblasts were cultured on native or 3DG-collagen. Nuclear extract was isolated and Sp1, c-fos, and c-myc activities were measured by ELISA according to the manufacturer's recommendations. We found the activities of all three transcripts that were measured were decreased on 3DG-collagen; Sp1 activity was decreased by 37% **(P = 0.0008),** c-fos by 30% **(P = 0.002)** and c-myc by 17% **(P = 0.01)**. These experiments have been performed 3 independent times and the values have been compiled

promoter fragments available in the laboratory) to determine if there is more binding of these transcription factors to the promoter. We have isolated the most active part of the promoter and was found to be 170 bases proximal to the start site of transcription. We will use this fragment in our analyses. Finally with the analysis of transcription factors, we will perform DNA affinity precipitation to identify other transcription factors that bind with Sp1, c-fos and c-Myc and potentially inhibit the transcription of COL1A1.

We went on to investigate the transcription factors that are the most important for collagen expression in SSc; Sp1, c-fos, and c-myc. We found that 3DG collagen decreased Sp1 transcription levels and Sp1 activity. Fibroblasts were cultured on 3DG-collagen or native collagen for 24 h and mRNA was purified, made into cDNA and Sp1, c-fos, and c-myc levels were assessed. We found that Sp1 mRNA transcripts were decreased by 88.7% ($p<0.0001$), c-fos was increased by 6% (not significant), and c-myc mRNA transcripts did not change (not significant). As Sp1 transcripts were decreased, we also investigated the transcription factor Sp3 by real-time PCR as we had previously demonstrated that Sp1 and Sp3 and able to interact to affect collagen gene regulation (12). We also found that Sp3 transcript levels were decreased by the 3DG-collagen; however, this decrease was modest (7%) and not statistically significant. We found that the changes to the activity levels of these proteins were not reflected by the alterations in the mRNA transcript numbers. This may be due to the increased degradation of these proteins in response to 3DG-collagen rather than alterations to transcription factor mRNA levels. We measured the activity of the proteins by ELISA assays in SSc fibroblasts and found that all the activities of the three transcripts were decreased; Sp1 activity was decreased by 37% ($p=0.0008$), c-fos by 30% ($p=0.002$) and c-myc by 17% ($p=0.01$)(Fig.14).

Electrophoretic Mobility Shift Assays. We know from previous work from my laboratory, the region spanning -174 to +1 in the collagen promoter is the most active part of the promoter of the COL1A1 gene (11). It contains two NF-1/Sp1 switch elements. The proximal NF-1/Sp1 switch element spans -107 to -64 and the distal switch site spans -174 to -107 bp upstream from the transcription start site. Therefore, we designed primers that isolated each of the switch elements individually and measured the binding of Sp1 to the DNA. We found that the distal part of the promoter affected collagen expression and was sensitive to the 3DG-collagen (Fig. 15). This is in keeping with our previously published data that demonstrated that the proximal NF-1/Sp1 had almost no promoter activity (11) and that the distal NF-1/Sp1 switch element modulated collagen expression. Note that there is no binding of protein to the distal promoter region with the nuclear extract from fibroblasts cultured on 3DG-collagen as evidenced by lack of a band. This suggests that there is a dramatic reduction in transcription factors, which we confirm in Fig. 14.

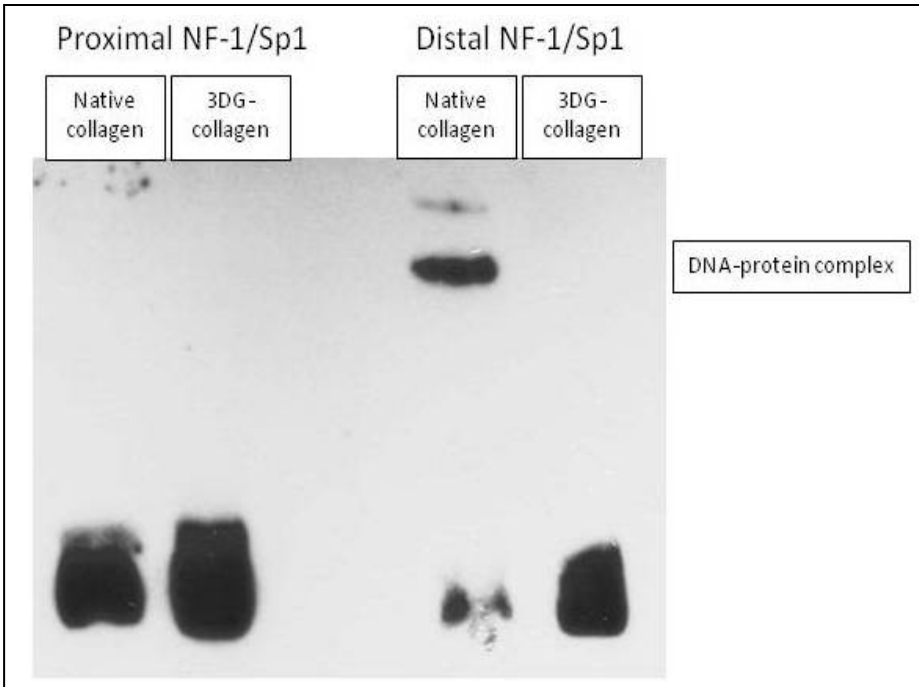

Figure 15. The Distal NF-1/Sp1 Switch Element is Involved in Decreased Collagen Expression observed in SSc Fibroblasts Cultured on 3DG-collagen. SSc fibroblasts were cultured on native or 3DG-collagen. Nuclear extract was isolated and incubated with biotin-labeled distal or proximal regions of the collagen promoter. The DNA-protein complexes were size fractionated on polyacrylamide gels, transferred to PVDF membrane and probed with an anti-biotin antibody. We found that the distal NF-1/Sp1 switch element was affected by the 3DG-collagen as noted by the band shift. This band shift was not observed with nuclear extract from fibroblasts cultured on 3DG-collagen or with the proximal NF-1/Sp1 switch site.

DNA Affinity Precipitation. We had originally planned to performed DNA affinity purifications; however, it became apparent to us while we were analyzing the electrophoretic mobility shift assays that no protein was binding to the region that held the highest transcriptional rate for type I collagen when fibroblasts were cultured on 3DG collagen (Fig. 15). We believe that this is due to the substantial depression of transcription factor activity (Fig. 14) as we saw a complete abolishment of the shifted band. If we had observed a slight shift remaining in the DNA protein complex in Fig. 14, we would have considered that another transcription factor has bound to this region and is inhibiting the expression of the collagen gene, however, we did not see this and therefore that no transcription factors are binding to this region when fibroblasts are cultured on 3DG-collagen.

3DG-collagen increases Smad7 expression. In an effort to further elucidate the signaling in fibroblasts cultured on 3DG-collagen that results in the decrease in COL1A1 and COL3A1 expression (Figs. 3, 5 & 7) and the fact that 3DG collagen decreases TGF-β1 protein (Fig. 8), we investigated the expression of Smads which are known to regulate TGF-β1 signaling. Smad7 expression is increased in fibroblasts cultured on 3DG-collagen and decreased on MG-collagen. TGF-β1 signals from the TGF-β1 receptor through the Smad pathway. The Smad pathway encompasses Smad3 heterodimerizing with Smad4 inducing their translocation to the nucleus where they interact with Smad-binding elements in promoters (e.g. collagen). Smad7 interferes with this heterodimerization, thus preventing the activation of Smad sensitive promoters (reviewed in reference 13). We found that Smad7 protein was induced in fibroblasts cultured on 3DG-collagen matrices, whereas it was decreased in fibroblasts cultured MG- collagen (Fig. 16). This data was published in Matrix Biology 2010.

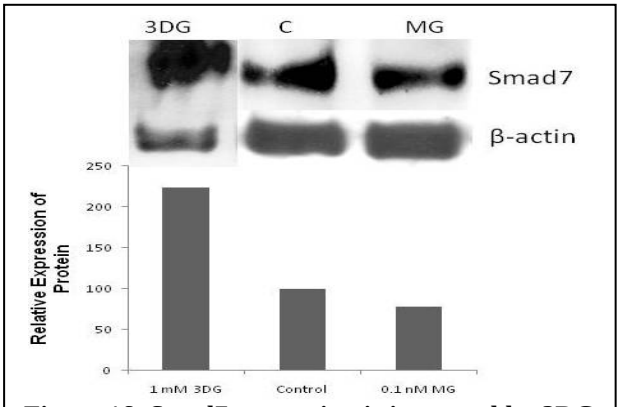

Figure 16. Smad7 expression is increased by 3DG modified collagen matrices and increased with MG modified collagen matrices. Fibroblasts were cultured on 1 mM 3DG collagen matrices or 0.1 nM collagen matrices for 24 h. Protein was extracted from the cells and size fractionated on polyacrylamide gels as described in the methods. SMAD7 expression was elevated in the fibroblasts cultured on 3DG-collagen matrices and decreased with MG collagen matrices, resulting in the inhibition or induction of collagen expression, respectively. 3DG = 3DG-modified collagen matrices, MG = MG-modified collagen matrices, C = native collagen with no modifications.

We have performed further experiments to try to elucidate the changes in signaling when fibroblasts are cultured on 3DG-collagen and how this translates to a decrease in collagen expression. The observations of decreased collagen expression in SSc fibroblasts cultured on 3DG-collagen cannot be fully accounted for with just the decreased transcription factors through the ERK1/2 pathway, therefore we investigated other signaling pathways from the integrin receptor. These following observations further define the role for 3DG in the reduced expression of collagen within the skin but also implicate the role of 3DG in diabetic complications such as chronic wounds.

Fibroblasts adhere more strongly to 3DG-collagen. We wanted to determine the binding strength of the fibroblast to the collagen fiber as this also induces feedback signals for collagen expression through the ERK pathway. Kinetic analyses have demonstrated that weakly adhesive surfaces induce fibroblasts to poorly adhere to the collagen and likewise, strongly adhesive surfaces can immobilize fibroblasts due to disruption of the cell-substratum attachments (14). Therefore, we measured the fibroblast's adhesive strength to the 3DG-collagen using a jet wash adhesion assay. Fig. 17A is a schematic representation of the number of cells binding to native collagen, 3DG-treated collagen, and aminoguanidine/3DG-treated collagen. Fibroblasts seeded onto native collagen begin to attach within 3 h, and this attachment is difficult to disturb by mechanical disruption (15). After 3 h a jet wash assay was performed and ten random fields of view were captured at 10X magnification and the numbers of cells in each field were counted. We found that an average of 280 cells/field remained attached on native collagen (Fig. 17B). In contrast, we observed an average of 353 cells/field remained on 3DG-collagen, representing an increase in cell adherence by 26% $\pm$ 1.41% (Fig. 17C; $p<0.0001$). Additional experiments with 5mM AG, which chelates 3DG prior to its reaction with the collagen, did not affect fibroblast binding to the collagen and an average of 276 cells/field remained with aminoguanidine (Fig. 17D) and native collagen compared to 283 cells/field with aminoguanidine and 3DG-collagen Fig. 17E). These findings could account for the decreased proliferation of fibroblasts when cultured on the 3DG-collagen as an increased binding strength to the collagen would not only inhibit the mobility of fibroblasts but also the

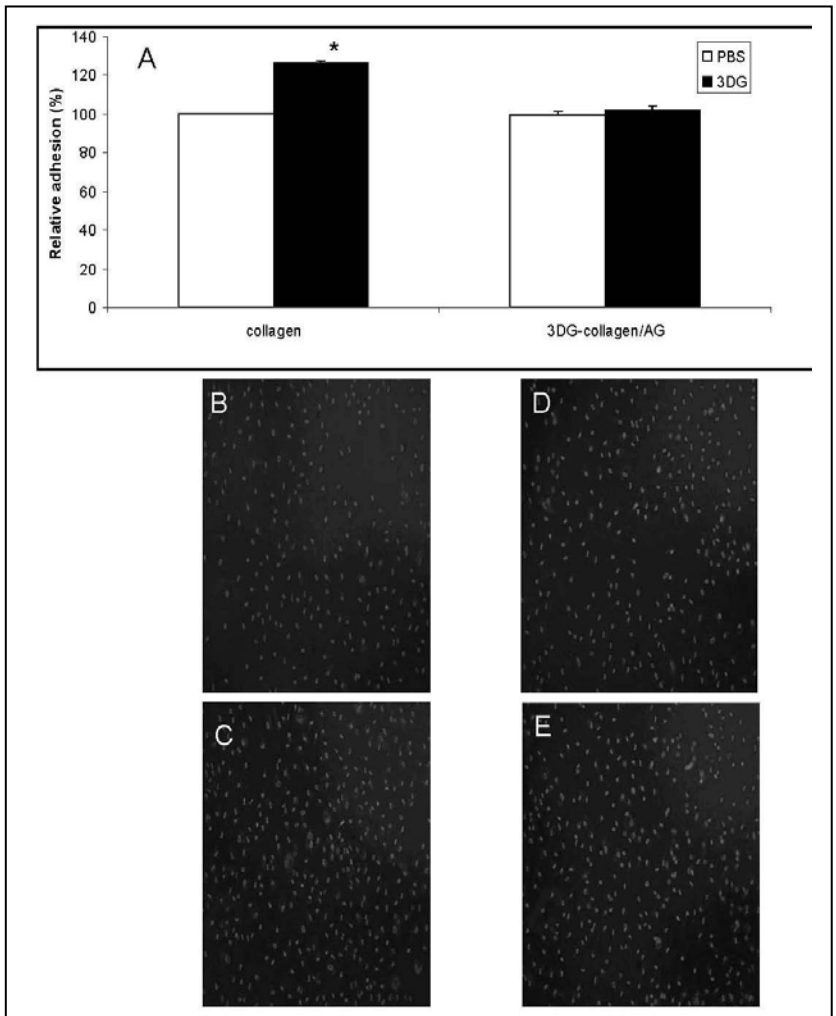

Figure 17. Effect of 3DG on Fibroblast Adhesion to Collagen. (A) Normal fibroblasts were seeded on native collagen or collagen cross-linked by 1mM 3DG and allowed to attach for 3h. In addition, aminoguanidine (AG) was added simultaneously with 3DG to chelate the 3DG. We estimated the collagen adhesive strength by counting the cells remaining after a jet wash assay according to Arnesen et al 2006. (A) Graphical representation of the binding strength of the fibroblasts to native collagen or the 3DG/AG-collagen. Cell numbers from 10 random images were counted and averaged. This experiment was performed on 3 separate occasions. (B-E) Show a representative image of the numbers of fibroblasts remaining after the jet wash assay. (B) Fibroblasts cultured on native collagen; (C) Fibroblasts cultured on 3DG-collagen; (D) Fibroblasts cultured on AG-collagen. (E) Fibroblasts cultured on AG-3DG-collagen. Fibroblasts were found to adhere 26% $\pm$1.4% more efficiently to the 3DG-collagen than the fibroblasts that were cultured on native collagen *$P < 0.03$. In the presence of AG, fibroblasts were found to adhere similarly to the native collagen (99% $\pm$ 2.1% for PBS and 102% $\pm$ 2.1% for 3DG treated). All images were taken at 10X magnification.

proliferation.

3DG-collagen induces the perinuclear localization of paxillin and focal adhesion kinase. In our control studies, we found several dramatic changes in fibroblasts when they were cultured in an environment of 3DG-collagen. Firstly, we found that fibroblasts had increased adhesion to 3DG-collagen and it is this increased adhesion to the collagen through integrins on the cell surface of the fibroblast that mediate downstream signaling. We found this to be surprising but when we further investigated this phenomenon, we found several abnormalities in protein expression of integrins, paxillin, and focal adhesion kinase was also altered. Integrins are the receptor for collagen and paxillin and focal adhesion kinase interact with the integrin receptor and transmit signals further downstream to the nucleus. We found that in fibroblasts cultured on 3DG-collagen, paxillin and focal adhesion kinase were located to the perinuclear region (Fig. 18). We speculate that changes in the localization of these proteins will directly affect signaling from the integrin receptor.

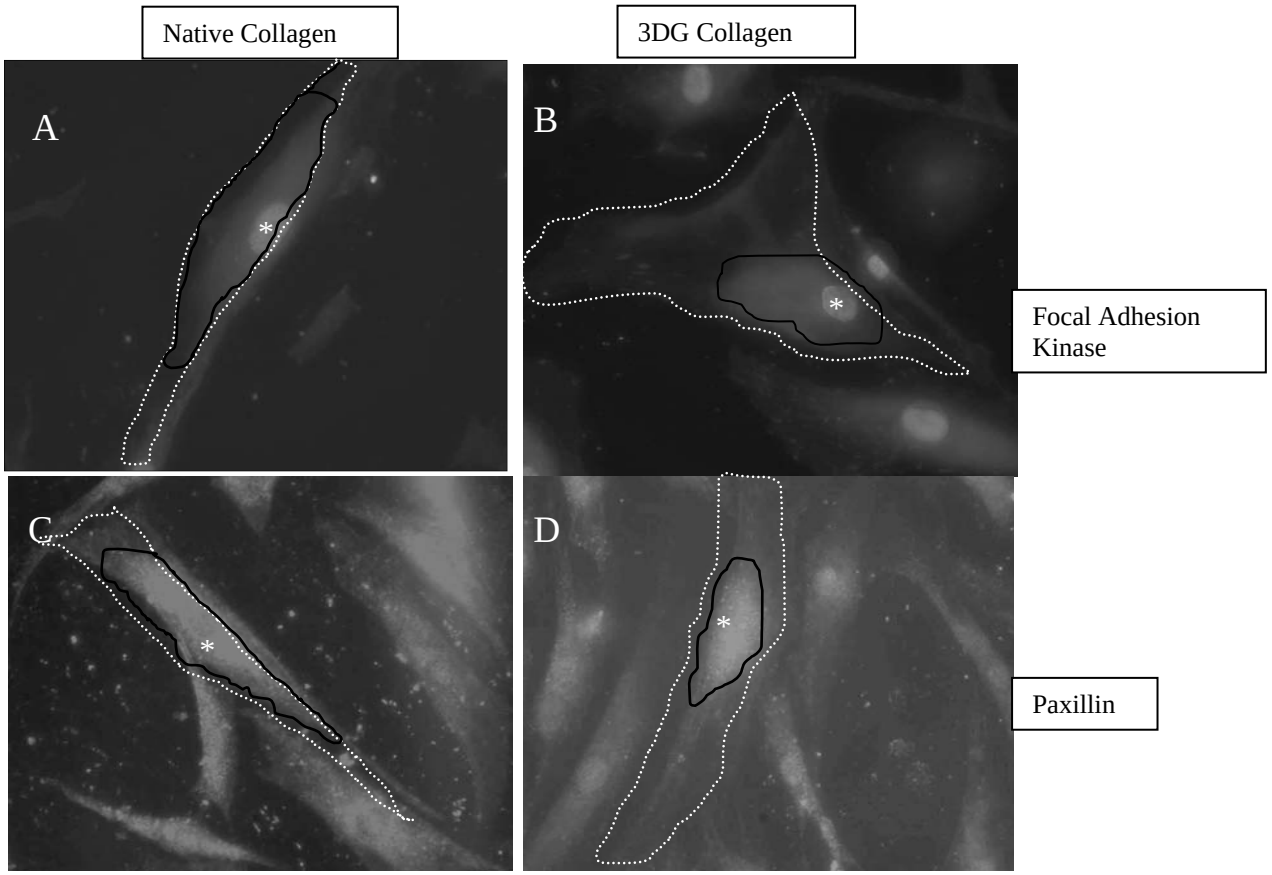

Figure 18. Localization of Paxillin and focal adhesion kinase in fibroblasts cultured on 3DG-collagen. Immunofluorescence of focal adhesion proteins, focal adhesion kinase and paxillin in subconfluent fibroblasts cultured on native collagen (A and C) and 3DG-collagen (B and D). Note the staining throughout the fibroblast for both focal adhesion kinase and paxillin when cultured on native collagen (A and C). However, when the fibroblasts were cultured on 3DG-collagen, the localization of focal adhesion kinase and paxillin shifted to the perinuclear region, away from the periphery of the fibroblast, closer to the nucleus (B and D). All images were taken at 40X magnification on an epi-fluorescence microscope. The nucleus is denoted by the white star, the periphery of the cell has been outline with the white dotted line and the primary location of the staining has been outlined with the black line.

These findings suggested to us that right from the periphery of the cell; the signaling has been altered as focal adhesion kinase and paxillin are now no longer located immediately under the integrin receptor where they are required to transmit signals to the nucleus. Because we found that ERK1/2 phosphorylation was decreased (Fig. 9), we believe that this change in protein localization has a direct effect on ERK1/2 signaling.

When we further investigated the expression of focal adhesion kinase and paxillin, we found that the expression of focal adhesion kinase was decreased by nearly 50%, whereas the expression of paxillin was not. Therefore, not only is the localization of focal adhesion kinase altered but the amount expressed is also changed (Fig. 19).

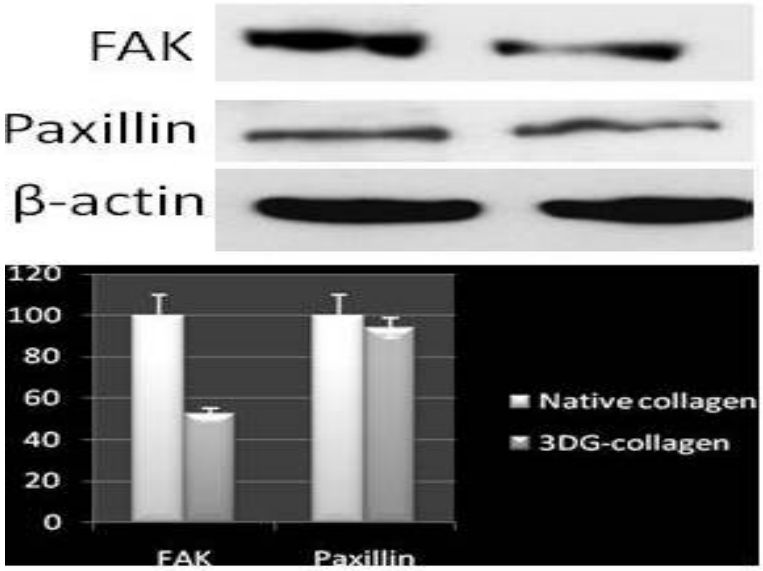

Figure 19. Decreased expression of focal adhesion kinase but not paxillin in fibroblasts cultured on 3DG-collagen. Fibroblasts were cultured on native collagen and 3DG–collagen. The PVDF was probed with an antibody directed against either focal adhesion kinase, paxillin, or β-actin. The intensity of the bands was measured by ImageJ and normalized to β-actin. It was found that focal adhesion kinase was decreased by approximately 50% in fibroblasts cultured on 3DG collagen **(P < 0.002),** whereas paxillin expression remained unaltered.

Specific Aim 2: Here, we will analyze the expression of integrins in response to the 3DG modified collagen matrices. Integrins are known to translate signals from the ECM via ERK to regulate cellular processes in the cell.

3DG-collagen modulates the expression of integrins. As we had protein available for the ERK1/2 and MEK1/2 western blotting experiments, we further investigated the expression of integrins as this proved to be more cost effective with regards to tissue culture reagents and employee time. Integrins are receptors for collagen and other

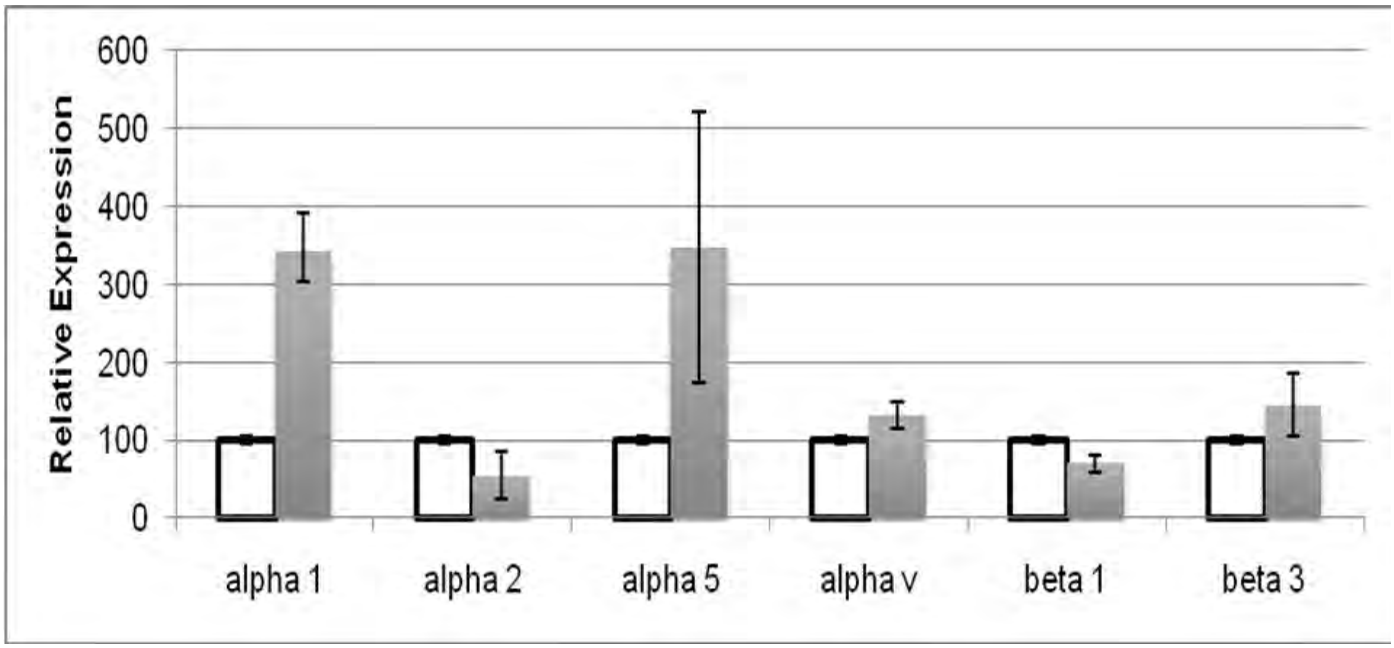

Figure 20. Alteration in mRNA expression of integrins on fibroblasts in response to 3DG-collagen. SSc fibroblasts were cultured on native collagen and 3DG-collagen, RNA was extracted and purified, and made into cDNA. The expression of integrins was measured by real-time PCR Comparing SSc fibroblasts cultured on native collagen to those cultured on 3DG-collagen; α1 integrin was increased by 3.5 fold **(P < 0.0001)**, α5 integrin by 3.5 fold **(P = 0.0058)**, αv integrin by 1.3 fold **(P = 0.0009)**, and β3 integrin by 1.5 fold **(P = 0.017)**; whereas α2 integrin was decreased by 50% **(P = 0.0053)** and β1 integrin was decreased by 30% **(P < 0.0001)**.

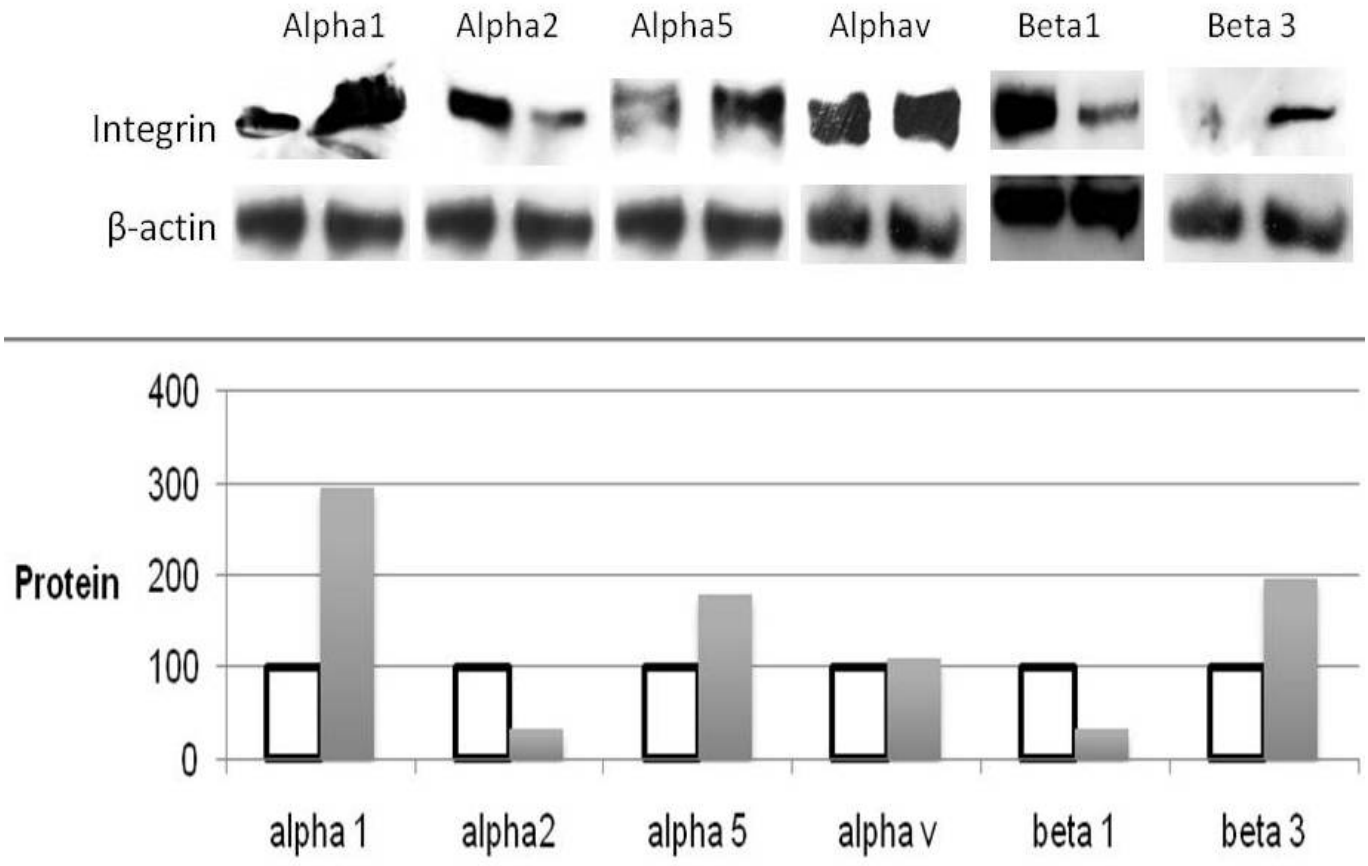

Figure 21. Alteration in protein expression of integrins on fibroblasts in response to 3DG-collagen. SSc fibroblasts were cultured on native collagen and 3DG-collagen, RNA was extracted and purified, and made into cDNA. The expression of integrins was measured by real-time PCR (A). Changes in integrin expression were also confirmed at the protein level (B) and graphed after corrections for β-actin as a control for protein loading (C).

extracellular matrix proteins and they are involved in outside-to-inside signaling in the fibroblast. They directly sense the immediate environment and alter the secretion of extracellular matrix proteins. We observed changes in integrin expression and some of the integrins were increased; whereas, others were found to be decreased (Fig. 20). Comparing SSc fibroblasts cultured on native collagen to those cultured on 3DG-collagen; α1 integrin was increased by 3.5 fold ($p<0.0001$), α5 integrin by 3.5 fold ($p=0.0058$), αv integrin by 1.3 fold ($p=0.0009$), and β3 integrin by 1.5 fold ($p=0.017$); whereas α2 integrin was decreased by 50% ($p=0.0053$) and β1 integrin was decreased by 30% ($p<0.0001$), (Fig. 20). Even though we found that mRNA transcripts were altered with 3DG-collagen, we then investigated changes in protein levels in fibroblasts cultured on the same matrix. Concordantly, we found that this also translated into changes in protein expression (Fig. 21). We found that α1 integrin was increased by 3 fold, α5 integrin by 3.5 fold, αv integrin by 1.3 fold, and β3 integrin by 1.5 fold; whereas α2 integrin was decreased by 50% and β1 integrin was decreased by 30% (Fig. 21).

Flow cytometry of fibroblasts reflected the level of expression found by Western blotting such that we found more surface expression of α1, β5, and β3 integrins and reduced levels of α2 and β1.

Fibroblasts require α1β1 to bind to 3DG-collagen. We further investigated the response of fibroblasts to the 3DG-collagen and determined which integrin receptor was the most important for the fibroblast adhesion to 3DG-collagen. β1 integrin is known to heterodimerize with α1 or α2 integrin, both of which are also important for cell adhesion and proliferation of fibroblasts on collagen networks (16-18). We therefore determined the direct contribution of β1, α1, and α2 integrins in 3DG-collagen binding by preincubating the fibroblasts with monoclonal antibodies targeting these integrins; and then culturing the preincubated fibroblasts on 3DG-treated collagen and performing the jet wash assay.

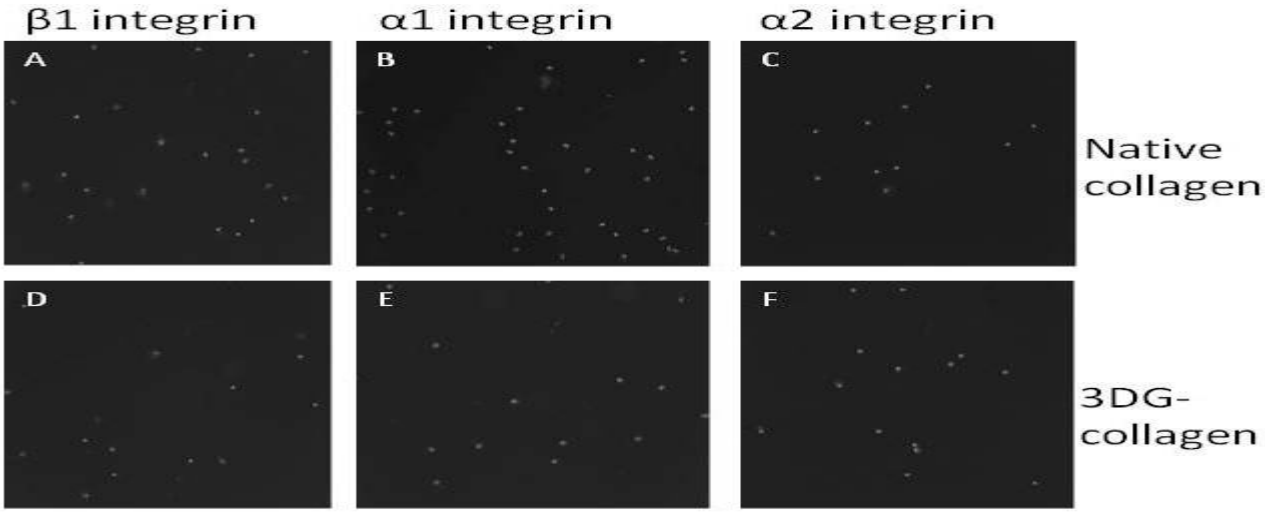

Figure 22. α1β1 integrin is involved in binding 3DG-collagen. Fibroblasts were coated with blocking antibodies for α1, α2, or β1 and allowed to attach to native or 3DG collagen. After 3 h, the fibroblasts were jet washed to remove the non-adherent cells and 10 fields of view were counted, as described above. α1β1 integrins were found to be involved in binding the 3DG-collagen, whereas α2 was not. On native collagen, blocking with β1 integrin, 45.5% ± 2.12% of the expected number of cells remained attached after the jet wash assay, 74% ± 1.41% remained with α1 integrin, and 53% ± 1.41% remained after blocking the α2 integrin (A-C; **P < 0.0001)**. With the 3DG-treated collagen, blockade of β1 integrin and α1 integrin induced a further reduction in adherence of cells to 3DG-collagen; 21% ± 2.12% of the expected cell number remained with β1 integrin and 48% ± 1.41% for α1 integrin (D-E; **P < 0.04)**. No difference was observed by blocking α2 integrin (F).

Antibodies to all three integrins decreased fibroblast attachment to native collagen: after incubation with β1 integrin, 45.5% ± 2.12% of the expected number of cells remained, with α1 integrin 74% ± 1.41% remained, and with α2 integrin 53% ± 1.41% of the expected number of cells remained attached to the collagen (Fig. 22A-C; p<0.0001). With the 3DG-treated collagen, blockade of β1 integrin and α1 integrin induced a further reduction in adherence of cells to 3DG-collagen; 21% ± 2.12% of the expected cell number remained with β1 integrin and 48% ± 1.41% for α1 integrin (Fig. 22D-E; p<0.04). No difference was observed by blocking α2 integrin (Fig. 22F). These data indicates that α1β1 integrin is essential for fibroblast adhesion to 3DG-collagen. This data was published in Wound Repair Regeneration, 2009.

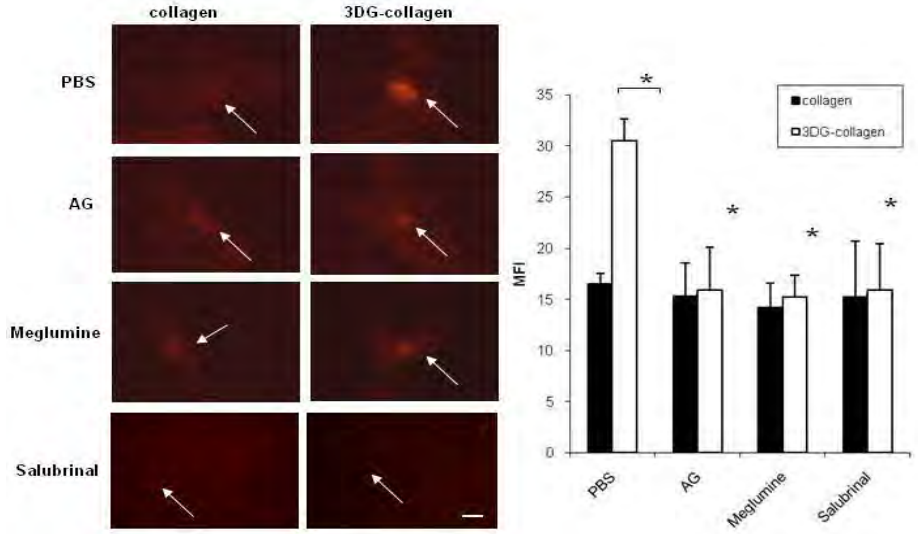

Figure 23. Effect of ER stress inhibitor salubrinal on 3DG-collagen-induced GADD153 expression. Fibroblasts were cultured in chamber slides coated with native collagen or 1 mM 3DG-collagen with or without 5 mM AG or 40 mM meglumine for 24 h. Also, fibroblasts were pretreated for 1 h with or without 40 μM salubrinal and then cultured on native collagen or 3DG-collagen for 24 h. Fibroblasts were stained and analyzed for expression of GADD153 in the nucleus by immunofluorescence analysis using Cy3-conjugated secondary antibody. Mean fluorescence intensity (MFI) of GADD153 in the nucleus was measured using ImageJ from ten representative fibroblasts. Images were taken at 40 X magnification on an epi-fluorescent microscope. Arrows indicate nuclei containing GADD153. The bars represent the MFI values from each experimental condition. Scale bar represents 10μm. ***p<0.0004**

3DG Increases the Expression of GADD153 in Fibroblasts. In addition to increased caspase-3 activity (Fig. 13), the presence of FAK and paxillin in the perinuclear region (Fig. 18) suggests that these proteins are being retained within the endoplasmic reticulum (ER) possibly due to protein misfolding (15). The characteristic marker for

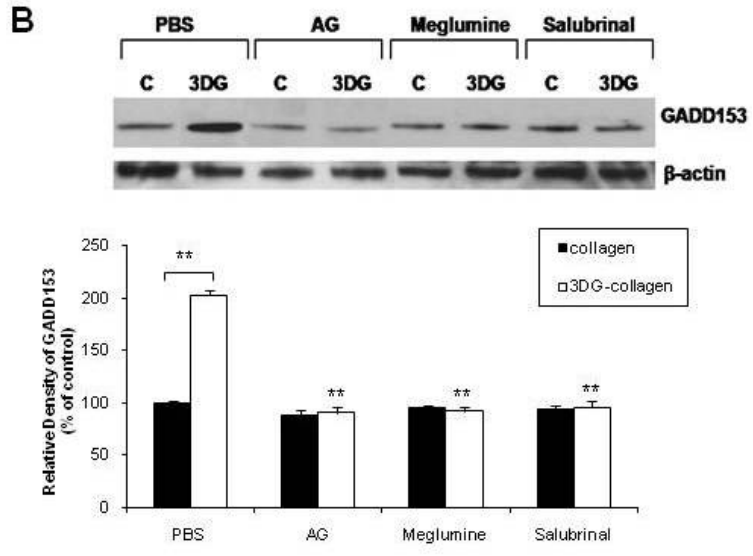

Figure 24. Effect of ER stress inhibitor salubrinal on 3DG-collagen-induced GADD153 expression. Fibroblasts were treated as in **A** followed by Western blot for GADD153 expression. β-actin was used as a loading control. The bars represent the densitometric value for each experimental condition. All comparisons are made against 3DG-collagen treated with PBS unless otherwise indicated. Data are mean ± SD (n=3), ****P < 0.0005.**

protein misfolding and ER stress is Growth Arrest and DNA Damage-Inducible Gene 153 (GADD153) (19). ER stress can occur due to the accumulation of membrane bound proteins in the ER, which in turn activates the transcription factor GADD153 (19). Under non-stress conditions, cells ubiquitously express GADD153 at very low levels in the cytosol; however during times of cellular stress, GADD153 is induced and accumulates in the nucleus (19). We hypothesized that the high levels of FAK and paxillin present in the perinuclear region of fibroblasts cultured on 3DG-collagen was due to improper protein folding. Transcript levels of GADD153 in fibroblasts cultured on 3DG-treated collagen was found to be increased by 83% ± 3.9%, P < 0.03 over fibroblasts cultured on native collagen (not shown).

Further confirming the elevation in GADD153 with 3DG-collagen, we calculated the mean fluorescent intensity (MFI) from histological analyses of fibroblast nuclei stained for GADD153 (Fig. 23). Histological analyses demonstrated that cells cultured on 3DG-collagen matrices had significant localization of GADD153 in the nucleus (31.3 MFI ± 4.641) compared to fibroblasts cultured on native collagen (17.7 MFI ± 2.307; Fig. 23). The differences in mean fluorescence intensity in fibroblasts cultured on 3DG-collagen vs. native collagen were significant, $p<0.0004$.

We confirmed the increased protein levels of GADD153 by western blotting and found that the induction of GADD153 was specific for 3DG-collagen because we inactivated 3DG with aminoguanidine (AG) and abolished the observed effect. Furthermore, we found that meglumine an inhibitor of 3DG and an inhibitor of ER stress (salubrinal) both abolished the effects of 3DG-collagen. This suggests that 3DG-collagen specifically induces ER stress and GADD153 protein (Fig. 24). To verify the immunofluorescence results, a Western blot was performed. Confirming the immunofluorescence observations, Western blot analysis demonstrated an increase to 203% ± 4.1% in GADD153 expression in fibroblasts cultured on 3DG-collagen compared to fibroblasts cultured on native collagen ($p<0.0005$). AG and meglumine reduced the level of GADD153 expression in fibroblasts cultured on 3DG-collagen to 91% ± 5.2% and 92% ± 4.2% ($p<0.0005$),

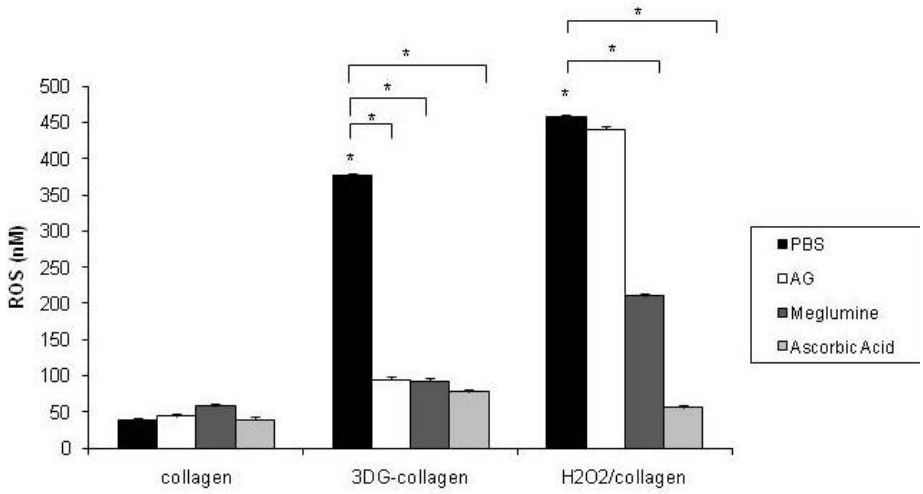

Figure 25. 3DG-collagen stimulates intracellular ROS in fibroblasts. Fibroblasts were cultured in 96-well plate coated with either native collagen or 1 mM 3DG-collagen or treated with or without 5 mM AG, 40 mM meglumine, or 100 µg/mL ascorbic acid for 24 h. Treatment of fibroblasts cultured on native collagen with 50 µM H_2O_2 was used as a positive control. Fibroblasts were loaded with DCFH-DA for 30 min and ROS production was measured by absorbance of fluorescent DCF at a wavelength of 480 nm/530 nm. Comparisons are made to collagen treated with PBS unless otherwise indicated. Data are mean ± SD (n=3), ***P < 0.001.**

respectively and salubrinal prevented the 3DG-collagen-induced increase of GADD153 ($p<0.0005$) (Fig. 24). These findings suggest that 3DG-collagen is inducing apoptosis through the ER stress-signaling pathway, which is dependent on GADD153 activation. This data was published in Plos One, 2010.

3DG-collagen stimulates ROS in dermal fibroblasts. ROS are known to cause oxidative stress and have been linked to the activation of GADD153-induced apoptosis in cells (20-22). Therefore, we determined if ROS were produced during the culturing of fibroblasts on 3DG-collagen. Fibroblasts cultured on 3DG-collagen produced 376 nM $\pm$ 3.4 of intracellular ROS at 24 h in comparison to the 38.7 nM $\pm$ 2.2 of ROS produced by fibroblasts grown on native collagen (Fig. 25, $p<0.001$). This increase was comparable to that observed with hydrogen peroxide (H_2O_2), a free radical involved in ER stress, which produced 458 nM $\pm$ 3.2 (Fig. 25, $p<0.001$). Moreover, AG abrogated the rise in ROS only in fibroblasts cultured on 3DG-collagen, and not in cells treated with H_2O_2 suggesting that 3DG-collagen is specifically producing ROS (Fig. 25; 94.3 nM $\pm$ 4.0 for 3DG-collagen/AG; 440.6 nM $\pm$ 4.0 for H_2O_2/AG, $p<0.001$). Meglumine inhibited the production of ROS in cells cultured on 3DG-collagen and partially inhibited ROS in cells treated with H_2O_2 suggesting that meglumine may prevent ROS induction by 3DG-collagen and/or may be a scavenger of free radicals (92.8 nM $\pm$ 4.0 for 3DG-collagen and meglumine; and 210.2 nM $\pm$ 4.4 for H_2O_2 and meglumine, $p<0.001$). In addition, the induction of ROS by fibroblasts cultured on 3DG-collagen, or treated with H_2O_2 could be blocked by pretreating fibroblasts with the antioxidant ascorbic acid (Fig. 25; 78.2 nM $\pm$ 3.6 for 3DG-collagen/ ascorbic acid; and 55.6 nM $\pm$ 4.8 for H_2O_2/ascorbic acid, $p<0.001$). Taken together, these results suggest that ROS could be produced in the fibroblast in response to the modification of collagen by 3DG. This data was published in Plos One, 2010.

NAD(P)H oxidase 4 is responsible for the 3DG-collagen-dependent production of ROS. The NAD(P)H oxidase (Nox) controls the production of ROS through integrin activation, and cytokine and growth factor stimulation (23-26). Overexpression of key oxidases such as the non-phagocytic Nox4 has been associated with increased ROS and apoptosis (23;24;27). Nox4 has been shown to be highly expressed in fibroblasts compared to other Nox homologues (23;24). Therefore, we determined if 3DG-collagen-induced ROS were mediated by the overexpression of Nox4. Quantitative real-time PCR revealed that Nox4 mRNA expression increased to 880% $\pm$ 200.0% in fibroblasts cultured on 3DG-collagen for 24 h compared to fibroblasts cultured on native collagen (Fig. 26, $p<0.02$). Moreover, to ensure that Nox4 was the only Nox isoform being over expressed by 3DG-collagen, quantitative real-time PCR was performed to determine the mRNA transcript levels of the other Nox isoforms, Nox1 and Nox2. Detection of Nox1 and Nox2 mRNA transcripts was not apparent suggesting that dermal fibroblasts over express specifically Nox4 (Fig. 26).

To show specificity of 3DG, AG and meglumine reduced the transcript levels of Nox4 in fibroblasts cultured on 3DG-collagen to that observed in fibroblasts cultured on native collagen. Additionally, Nox4 protein levels were found to be increased in fibroblasts cultured on 3DG-collagen compared to fibroblasts

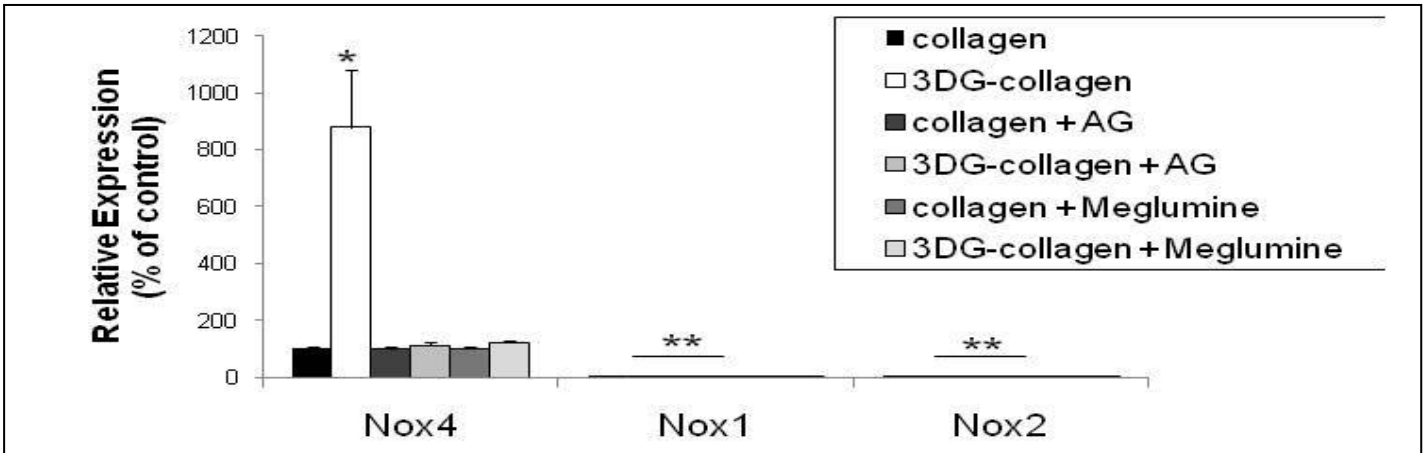

Figure 26. 3DG-collagen increases expression of Nox4 in the dermal fibroblast. Fibroblasts were cultured on either native collagen or 1 mM 3DG-collagen and treated with or without 5 mM AG or 40 mM meglumine for 24 h. **A**, Nox4, Nox1, and Nox2 mRNA expression levels were quantified by real-time PCR. All transcripts were normalized to β-actin. ***p<0.02, **p<0.001**

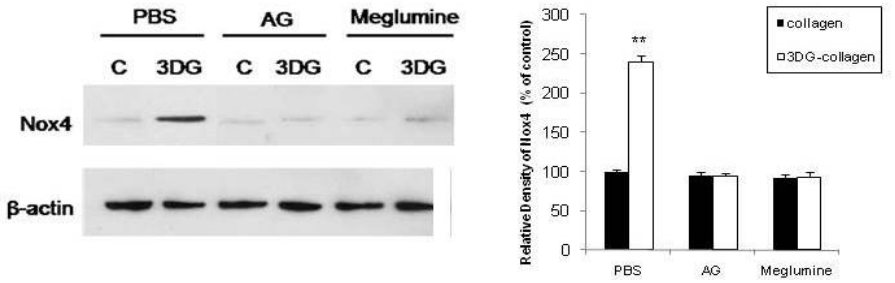

Figure 27. 3DG-collagen increases protein levels of Nox4. Expression levels of Nox4 were analyzed by Western blot and β-actin served as a loading control. Results were quantified by densitometric scanning of the Western blot and normalized for β-actin. ****p<0.001**

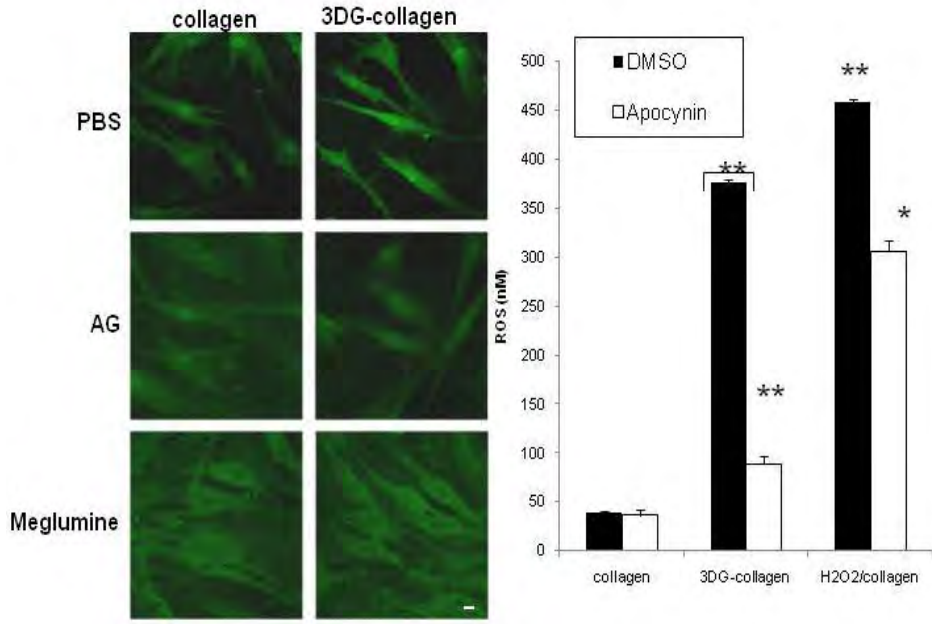

Figure 28. Immunofluorescence of Nox4. Localization of Nox4 in fibroblasts treated the same as in **Fig. 27** was analyzed by immunofluorescence with the anti-Nox4 polyclonal antibody and Cy2-conjugated secondary antibody. Images were taken at 40 X magnification on an epi-fluorescence microscope. Scale bar represents 10μm. Inhibition of Nox4 with apocynin reduces the level of intracellular ROS. Fibroblasts were pretreated for 1 h with either vehicle, DMSO or NOX inhibitor, apocynin (1 mM) and cultured on native collagen, 1 mM 3DG-collagen, or cultured on native collagen and treated with 50 μM H_2O_2 for 24 h. Fibroblasts were then incubated with DCFH-DA for 30 min and the level of intracellular ROS was determined by measuring the fluorescence at 480 nm/530 nm. Comparisons are made against collagen treated with DMSO and/or PBS unless otherwise indicated. Data are mean ± SD (n=3), ****P<0.001, *P < 0.02.**

cultured on native collagen (240% ± 8.6% in 3DG-collagen treated vs. 100% ± 3.3% in native collagen treated cells, Fig. 27, p<0.001). This upregulation was also abrogated by the 3DG inhibitors AG and meglumine.

Immunofluorescence demonstrated increased Nox4 localization at the plasma membrane in fibroblasts cultured on 3DG-collagen and that this increase was abrogated by AG and meglumine, suggesting that Nox4 may be activated on the cell surface (Fig. 28). We further investigated the role of Nox4 in the upregulation of ROS in fibroblasts cultured on 3DG-collagen. Fibroblasts were pretreated with apocynin, a broad class Nox inhibitor, cultured on either native collagen, 3DG-collagen, or treated with H_2O_2 for 24 h, and intracellular ROS was quantified. Apocynin reduced ROS in fibroblasts cultured on 3DG-collagen to that observed in fibroblasts cultured on native collagen (Fig. 28, p<0.001). This further confirms that 3DG-collagen is inducing ROS through activation of Nox4. Furthermore, apocynin was found to only partially inhibit the level of ROS in fibroblasts cultured on native collagen with H_2O_2 suggesting that apocynin is inhibiting the ROS induced by the Nox4 complex rather than affecting the induction of ROS by exogenous H_2O_2 (Fig. 28, p<0.02). This data was published in Plos One, 2010.

3DG-collagen-induced phosphorylation of p38 MAPK is dependent on upstream ROS. During times of ER stress, ROS have been shown to activate the stress kinase p38 MAPK (20;28;29). To determine if 3DG-collagen-induced ROS are responsible for increased phosphorylation of p38 MAPK, fibroblasts were pretreated with the antioxidant ascorbic acid and the Nox inhibitor apocynin, and cultured on either native collagen, 3DG-collagen, or native collagen and treated with H_2O_2 and protein levels were measured by Western blotting. Fibroblasts cultured on 3DG-collagen increased the phosphorylation of p38 MAPK to 175% ± 4.1%. As a positive control for ROS-induced p38 MAPK activation, fibroblasts cultured on native collagen and treated with H_2O_2 increased the phosphorylated p38 MAPK to 181% ± 3.3%. Pretreatment with ascorbic acid reduced the phosphorylation of p38 MAPK in fibroblasts cultured on 3DG-collagen or treated with H_2O_2 to that seen in

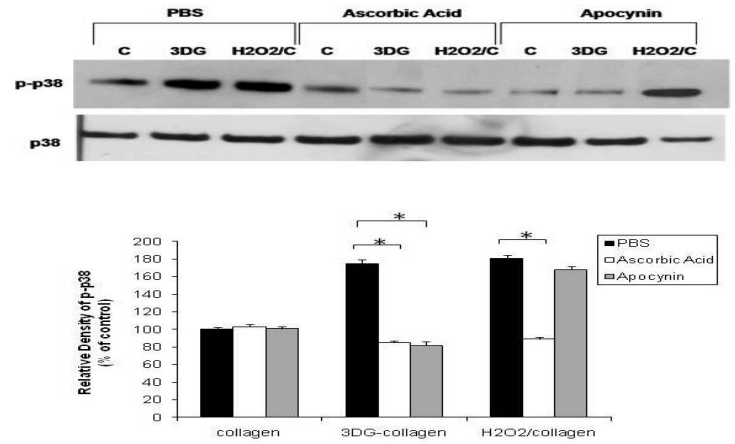

Figure 29. Phosphorylation of p38 MAPK is dependent on 3DG-collagen-induced ROS. Fibroblasts were pretreated for 1 h with 100 µg/mL of ascorbic acid, 1mM apocynin, or DMSO and cultured on either native collagen, 1 mM 3DG-collagen, or treated with 50 µM H_2O_2 for 24 h. Whole cell lysates were extracted and Western blot analysis of p-p38 MAPK was performed. Total p38 MAPK was used as a loading control. The bars correspond to the densitometric value of p-p38 MAP kinase after normalization for total p38 MAP kinase. Data are mean ± SD (n=3), ***P < 0.0001.**

fibroblasts cultured on native collagen (Fig. 29, $p<0.0001$). Additionally pretreatment with apocynin reduced the level of phosphorylated p38 MAPK in fibroblasts cultured on 3DG-collagen, but not in fibroblasts treated with H_2O_2 (Fig. 29, $p<0.0001$). These results suggest that 3DG-collagen-induced p38 MAPK is dependent on upstream production of ROS by Nox4. This data was published in Plos One, 2010.

3DG-collagen-induced GADD153 expression is dependent on upstream ROS and p38 MAPK activation. 3DG-collagen-induced ROS can lead to phosphorylation of p38 MAPK, which is essential for the activation of GADD153; therefore, the functional role of ROS and p38 MAPK in GADD153 induction was assessed (20;21;28). To determine whether GADD153 induction by 3DG-collagen was a result of free radical-mediated effects, fibroblasts were pretreated with ascorbic acid or apocynin and then cultured on native collagen or 3DG-collagen for 24 h. Fibroblasts cultured on native collagen and treated with H_2O_2 were used as a positive control for ROS-induced GADD153 activation. The trafficking of GADD153 from the cytosol to the nucleus was found to be downregulated to 14.6 MFI ± 2.1 and 16.3 MFI ± 0.98 in response to ascorbic acid in fibroblasts cultured on 3DG-collagen or native collagen treated with H_2O_2, respectively (Fig. 30, $p<0.007$). The expression of GADD153 in the nucleus of fibroblasts pretreated with

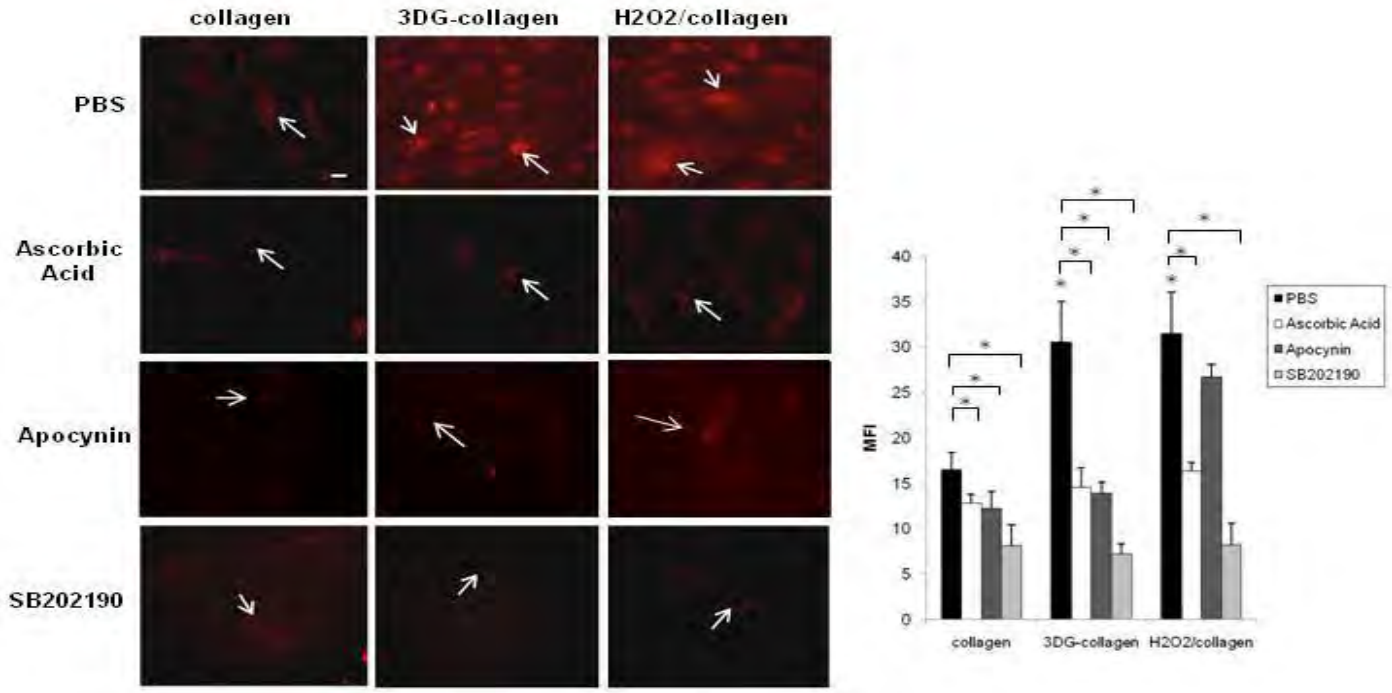

Figure 30. Inhibition of ROS, and p38 MAPK abrogates GADD153 expression in fibroblasts cultured on 3DG-collagen. Fibroblasts were pretreated with 100 µg/mL of ascorbic acid, 1mM apocynin, or 10 µM SB202190 for 1 h, and cultured on native collagen, 1 mM 3DG-collagen or treated with 50 µM H_2O_2 for 24 h. **A**, Fibroblasts were stained and analyzed for the expression of GADD153 in the nucleus by immunofluorescence using a Cy3-conjugated secondary antibody. Representative images were taken at 40 X magnification on an epi-fluorescence microscope, and the MFI of ten nuclei was analyzed by Image J. Bars correspond to the MFI of treated fibroblasts. Arrows indicate nuclei containing GADD153. Scale bar represents 10µm. ***P < 0.007.**

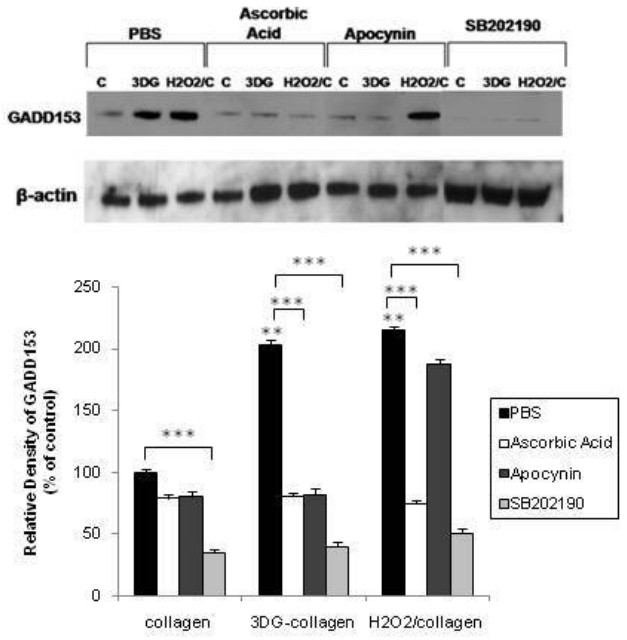

Figure 31. GADD153 Expression requires p38MAPK. Western blot for the expression of GADD153 in whole cell lysates. β-actin was used as a loading control. The bars correspond to the densitometric value of GADD153 after normalization for β-actin. All comparisons are made against collagen treated with PBS unless otherwise indicated. Data are mean ± SD (n=3), *****P<0.0001, **P < 0.007.**

inhibition of p38 MAPK reduced the level of GADD153 expression in fibroblasts grown on native collagen treated with H_2O_2 to 51% ± 3.6% (Fig. 31, p<0.0001), and its nuclear localization to 8.21 MFI ± 2.4 (Fig. 31, p<0.007). These results suggest that the induction of p38 MAPK by upstream ROS is responsible for the activation of GADD153 by 3DG-collagen. This data was published in Plos One, 2010.

3DG-collagen-induced caspase-3 activation is dependent on upstream ROS and p38 MAPK activation. A caspase-3 assay was performed to determine if ROS and p38 MAPK are responsible for the increased caspase-3 activation observed in fibroblasts cultured on 3DG-collagen. Fibroblasts were pretreated with ascorbic acid, the Nox inhibitor apocynin, or the p38 MAPK inhibitor SB202190; and cultured on native collagen or 3DG-collagen for 24 h, or cultured on native collagen and treated with H_2O_2 as a positive control. Fibroblasts treated with ascorbic acid, apocynin, or SB202190 and cultured on 3DG-collagen reduced the activation of caspase-3 to apocynin and cultured on 3DG-collagen was also reduced to 13.9 MFI ± 1.2 (Fig. 30, p<0.007). Western blot was performed to verify the expression of GADD153. GADD153 expression was decreased in response to ascorbic acid and apocynin in fibroblasts cultured on 3DG-collagen, while only ascorbic acid reduced the level of GADD153 in fibroblasts cultured on native collagen treated with H_2O_2 (Fig. 30; 81% ± 2.4% 3DG-collagen treated with ascorbic acid and 82% ± 4.6% treated with apocynin, and 75% ± 2.2% H_2O_2 treated with ascorbic acid and 188% ± 3.6% treated with apocynin, p<0.0001). These results suggest that the generation of ROS by Nox4 lies upstream of GADD153.

Next, the role of p38 MAPK in GADD153 activation was assessed in fibroblasts cultured on native collagen or 3DG-collagen. Fibroblasts were pretreated with the p38 MAPK inhibitor SB202190 and cultured on native collagen, 3DG-collagen, or native collagen and treated with H_2O_2 for 24 h. Inhibition of p38 MAPK by SB202190 reduced the localization of GADD153 in the nucleus to 7.23 MFI ± 1.13% in fibroblasts cultured on 3DG-collagen (Fig. 31, p<0.007), and reduced the expression of GADD153 to 40% ± 4.0% (Fig. 31, p<0.0001). In addition,

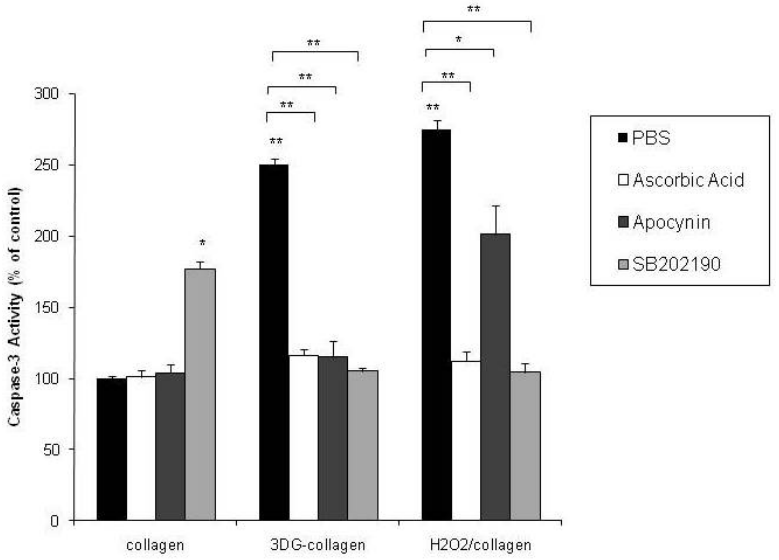

Figure 32. Inhibition of ROS, and p38 MAPK reduces caspase-3 cleavage induced by 3DG-collagen. Fibroblasts were pretreated with 100 µg/mL of ascorbic acid, 1mM apocynin, or 10 µM SB202190 for 1 h and cultured on native collagen, 1 mM 3DG-collagen, or treated with 50 µM H_2O_2 for 24 h. Treatment of fibroblasts with 50 µM H_2O_2 for 24 h was used as a positive control. 100 µg of whole cell lysate was assayed for caspase-3 activity according to the protocol from Caspase-3 Colorimetric Correlate Assay. All samples were performed in triplicate and normalized to the control samples. All comparisons are made against collagen treated with PBS unless otherwise indicated. Data are mean ± SD (n=3), ****P < 0.0002, *P < 0.001.**

116% ± 4.7%, 115% ± 4.5%, and 105% ± 2.5% respectively. This expression was comparable to the level of caspase-3 cleavage observed in fibroblasts cultured on native collagen and treated with H_2O_2 in the presence of ascorbic acid (112% ± 7.2%) or SB202190 (104.2% ± 6.6%), and fibroblasts cultured on native collagen (100% ± 1.4%; Fig. 32, $p<0.0002$). This data suggests that 3DG-collagen is inducing caspase-3 activation through ER stress, which is dependent on upstream activation of ROS and p38 MAPK through upregulation of Nox4. This data was published in Plos One, 2010.

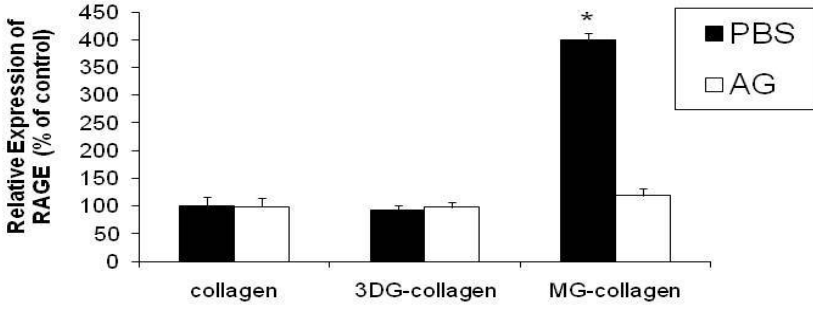

Figure 33. 3DG-collagen does not induce the expression of RAGE. A, Fibroblasts were cultured on native collagen, 1 mM 3DG-collagen, or 1 mM MG-collagen with or without 5 mM AG for 24 h. mRNA was analyzed for the expression of RAGE by real-time PCR. All transcripts were normalized to β-actin. Data are mean ± SD (n=3), ***P < 0.002.**

3DG-collagen induces ROS and apoptosis independent of RAGE signaling. When AGEs bind to their receptor, RAGE, ROS are released as a downstream byproduct (30). To determine if 3DG-collagen is signaling via its interaction with RAGE, we investigated transcript levels of total RAGE. To our surprise, 3DG-collagen did not upregulate RAGE transcript levels (Fig. 33). As a control for the induction of RAGE we cross-linked collagen with MG, which is a well studied AGE precursor known to signal via RAGE (31;32). We observed a significant induction of RAGE transcript levels in fibroblasts cultured on MG-collagen; 400% ± 12% upregulation of RAGE (Fig. 33, $p<0.002$). Additionally, treatment of MG-collagen with AG reduced the transcript levels of RAGE to 119% ± 13.3%, which confirms that MG can upregulate RAGE mRNA expression. To ensure that 3DG-collagen is not regulating the RAGE receptor post-transcriptionally we measured RAGE protein levels by Western blot. In contrast to the 238% ± 11.8% upregulation of RAGE protein in fibroblasts cultured on MG-collagen, 3DG-collagen did not upregulate RAGE protein expression in fibroblasts (107% ± 10.5%; Fig. 34). To further confirm the specificity of MG, AG abrogated the increase in RAGE protein expression (104% ± 8.2%) in fibroblasts cultured on MG-collagen (Fig. 34, $p<0.002$). This data suggests that 3DG-collagen is not upregulating RAGE at both the level of transcription or translation.

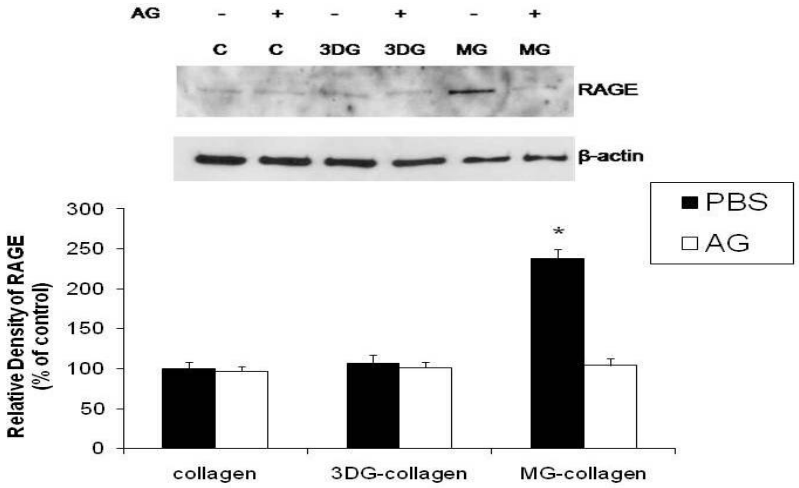

Figure 34. RAGE protein is not induced by 3DG-collagen. Fibroblasts were treated as in **A** and analyzed for the expression of RAGE by Western blot. The bars correspond to the densitometric value of RAGE after normalization for β-actin. All comparisons are made against collagen treated with PBS. Data are mean ± SD (n=3), ***P < 0.002**.

To verify the absence of RAGE expression in 3DG-collagen signaling, RAGE was blocked using a blocking antibody specific for the extracellular domain of RAGE, and the levels of ROS were quantified. Intriguingly, the levels of ROS in fibroblasts cultured on 3DG-collagen did not alter after blockade of RAGE. However, we observed the downregulation of ROS with the inhibition of RAGE binding in fibroblasts cultured on MG-collagen (Fig. 35A, $p<0.001$). To demonstrate that 3DG-collagen signaling was independent of RAGE, we investigated the expression of GADD153. Fibroblasts were pretreated with the RAGE blocking antibody and cultured on native collagen, 3DG-collagen, or MG-collagen; and the level of activated GADD153 was quantified. Blockade of RAGE in fibroblasts cultured on 3DG-collagen did not suppress the activation of GADD153, while GADD153 was suppressed after blockade of RAGE in fibroblasts cultured on MG-collagen

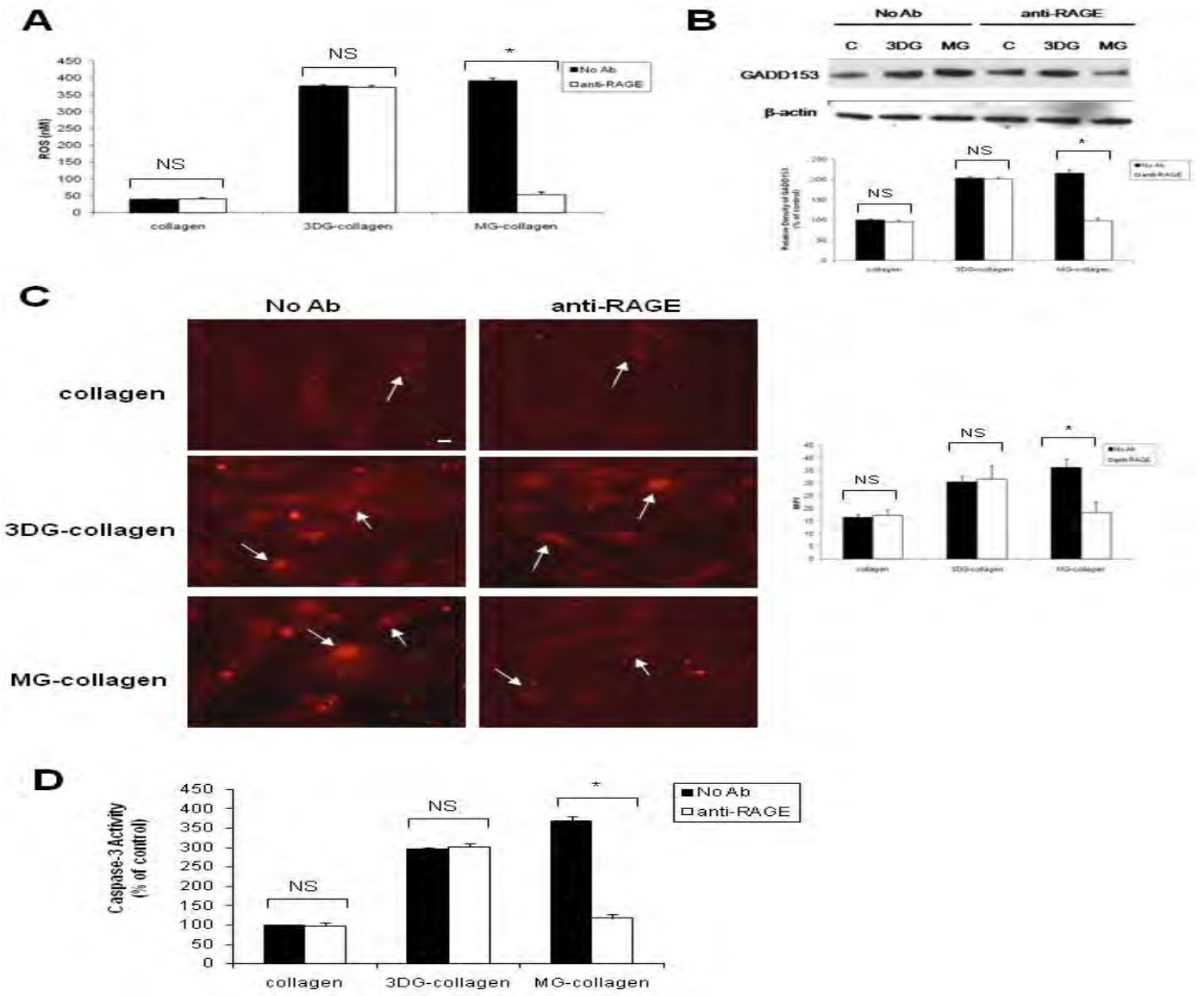

Figure 35. Inhibition of RAGE does not alter the induction of ER stress pathway in fibroblasts cultured on 3DG-collagen. **A**, Fibroblasts were pretreated with or without the blocking antibody anti-RAGE (10 μg/μL) for 1 h and cultured on native collagen, 1 mM 3DG-collagen, or 1 mM MG-collagen for 24 h and analyzed for the production of ROS. Fibroblasts were incubated with DCFH-DA for 30 min and the level of intracellular ROS was determined by measuring the fluorescence at 480 nm/530 nm. **B**, Western blot of GADD153 expression after inhibition of RAGE. The bars correspond to the densitometric value of GADD153 after normalization for β-actin. **C**, GADD153 localization in the nucleus was analyzed by immunofluorescence using a Cy3-conjugated secondary antibody. Images were taken at 40X magnification on an epi-fluorescence microscope and the MFI of ten nuclei was processed by ImageJ. Arrows indicate nuclei containing GADD153. Scale bar represents 10μm. **D**, Caspase-3 activity analyzed according to the protocol from Caspase-3 Colorimetric Correlate Assay. All samples were performed in triplicate and normalized to the control samples. Data are mean ± SD (n=3), ***P < 0.001.**

(Fig. 35B-C, p<0.001). Blockade of RAGE did not decrease the level of caspase-3 activity in fibroblasts cultured on 3DG-collagen, while suppression of caspase-3 activity was observed in fibroblasts pretreated with the RAGE antibody and cultured on MG-collagen (Fig. 35D, p<0.001). These results suggest that 3DG-collagen is not signaling through the RAGE receptor as is observed with MG. This data was published in Plos One, 2010.

3DG-collagen activates the ER stress signaling cascade through α1β1 integrin. To delineate the receptor involved in activating the ER stress pathway by 3DG-collagen, we investigated α1β1 integrin collagen receptor. Previous data has demonstrated that fibroblasts have an increased adherence to 3DG-collagen, which is dependent on binding by α1β1 integrin (33). Fibroblasts can change their binding affinity for 3DG-collagen, which may cause an overproduction of ROS resulting in increased caspase-3 activation. To verify the role of

α1β1 integrin on the ER stress signaling pathway, fibroblasts were pretreated with blocking antibodies against either β1 or α1 integrin and the level of ROS was quantified. α5 integrin, the alpha subunit responsible for binding fibronectin, was used as a negative control. Neutralization of both β1 and α1 integrin reduced the production of ROS in fibroblasts cultured on 3DG-collagen to that seen in fibroblasts cultured on native collagen, while neutralization of α5 integrin did not affect the production of ROS (Fig. 36A, $p<0.001$). We next investigated the effect of β1 and α1 integrin neutralization on the expression of GADD153 in fibroblasts cultured on native collagen or 3DG-collagen for 24 h. Blockade of both β1 and α1 integrins suppressed the activation of GADD153 as seen by decreased protein expression and nuclear localization (Fig. 36B-C,

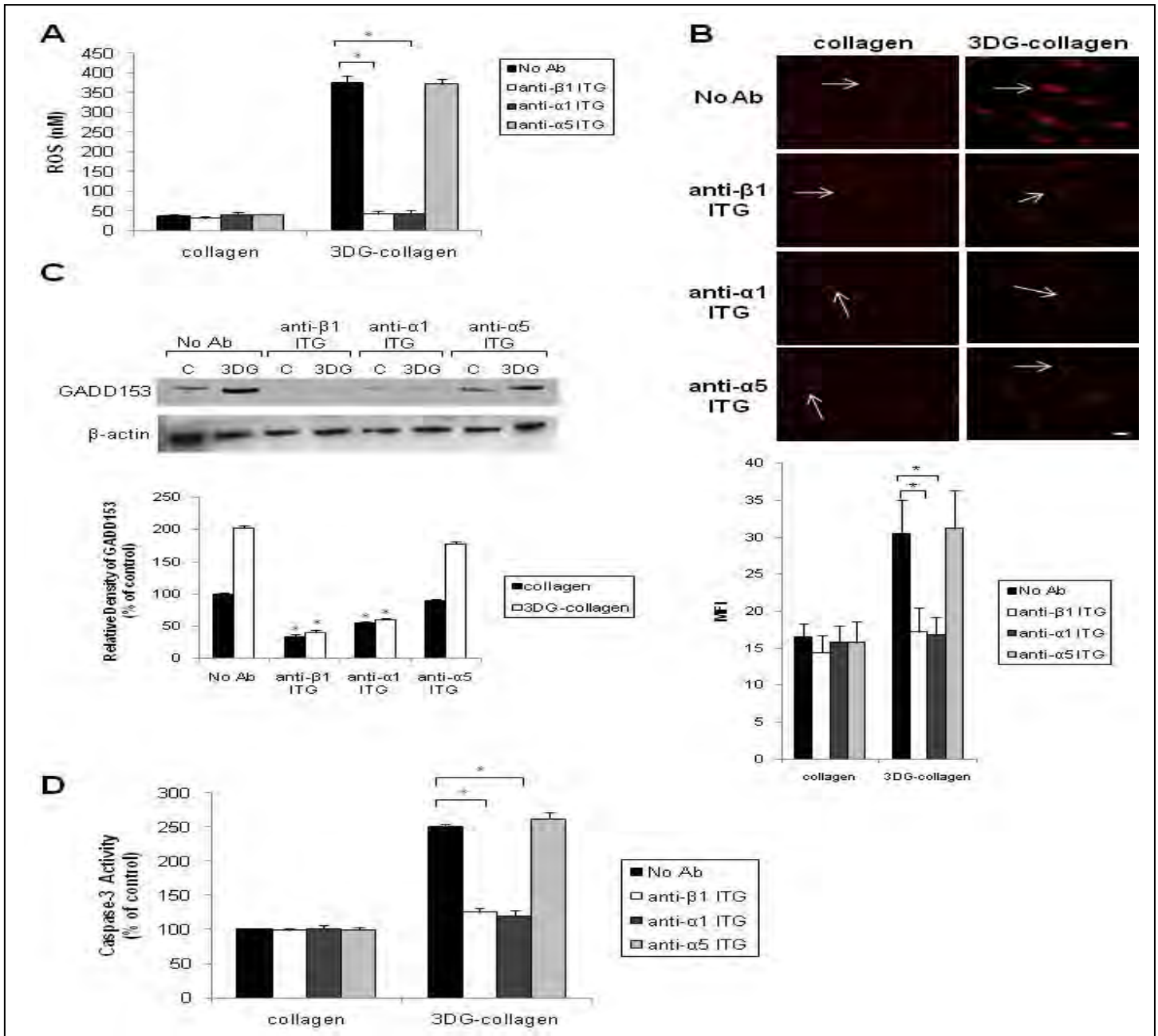

Figure 36. Effect of neutralization of α1β1 integrin on 3DG-collagen-induced ER stress pathway. **A**, Fibroblasts were pretreated with or without the blocking antibodies anti-β1 ITG, anti-α1 ITG, and anti-α5 ITG (10 μg/μL) for 30 min and cultured on native collagen, or 1 mM 3DG-collagen, for 24 h and analyzed for the production of ROS. Fibroblasts were incubated with DCFH-DA for 30 min and the level of intracellular ROS was determined by measuring the fluorescence at 480 nm/530 nm. **B**, GADD153 localization in the nucleus was analyzed by immunofluorescence using a Cy3-conjugated secondary antibody. Images were taken at 40X magnification on an epi-fluorescence microscope and the MFI of ten nuclei was processed by ImageJ. Arrows indicate nuclei containing GADD153. Scale bar represents 10μm. **C**, Western blot of GADD153 expression after neutralization of β1, α1, and α5 integrins. The bars correspond to the densitometric value of GADD153 after normalization for β-actin. **D**, Caspase-3 activity detected using the Caspase-3 Colorimetric Correlate Assay. All comparisons are made against collagen treated with PBS unless otherwise indicated. Data are mean ± SD (n=3), ***P < 0.001.**

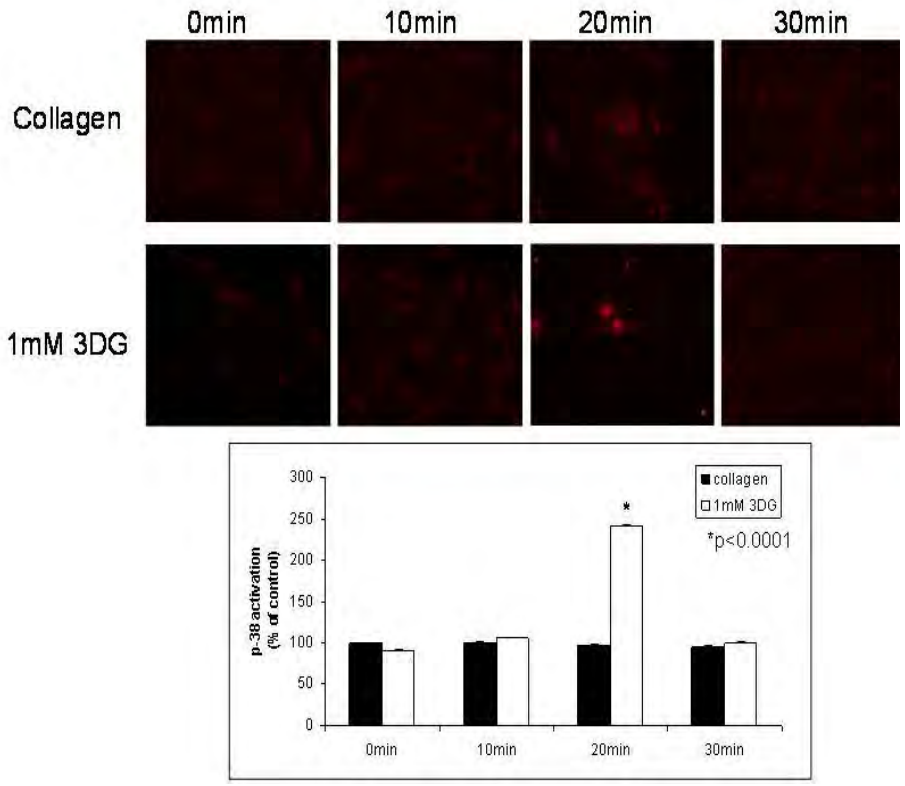

Figure 37. Increased localization of phospho-p38 MAP kinase in nuclei cultured on native and 3DG-collagen. Fibroblasts were cultured on native collagen in chamber slides and 3DG was added. Cells were fixed at specific time intervals and stained for phospho-p38. The intensity of the staining in the nucleus was quantified by ImageJ. The expression of phosphor-p38 protein was found to be significantly increased in the fibroblasts cultured on 3DG-collagen ***P < 0.0004** and that the signal peaked in the nucleus at 20 min.

p<0.001). Moreover, neutralization of β1 and α1 integrin in fibroblasts cultured on 3DG-collagen reduced the activity of caspase-3 to that observed in fibroblasts cultured on native collagen (Fig. 36D, p<0.001). These results suggest that the increased binding affinity of α1β1 integrin to 3DG-collagen causes the overproduction of ROS, which in turn leads to increased GADD153 activation and cleavage of caspase-3. This data was published in Plos One, 2010.

Phosphorylated p38 MAP kinase is transient and reaches a peak at 20 mins post exposure to 3DG. GADD153 expression is modulated by the phosphorylation of p38 MAP kinase contributing to the increased expression of caspase-3. This current study has established that in vitro exposure of human fibroblasts to 3DG-collagen leads to a reduction of key focal adhesion proteins and upregulation of GADD153. This inhibition and relocalization of focal adhesion proteins could be due to the highly dynamic interaction between integrins and the 3DG-collagen, which induced the stronger adherence of the fibroblast to the collagen matrix. Indeed, misfolding of focal adhesion proteins could cause both upregulation of GADD153, and down-regulation of ERK1/2. As the p38 MAP kinase protein is involved in modulating signals from focal adhesion kinase, we sought to determine if there were changes in the phosphorylation of p38 MAP kinase due to the 3DG-collagen. There was induction of p38 MAP kinase in the nucleus in fibroblasts cultured on 3DG-collagen compared to native collagen. We also found that the signal was fleeting and peaked in the nucleus at 20 min and was gone (Fig. 37).

HRAS expression inversely correlates with ASK1 expression. p38 MAPK has been shown to be a key signaling molecule for both HRAS as well as ASK1 (34-37). Evidence has suggested that HRAS activates p38 MAPK to induce cell motility and proliferation (38;39); while, ASK1 activates p38 MAPK under times of stress to induce apoptosis (37;40;41). Because 3DG-collagen is known to induce apoptosis through activation of p38 MAPK (33), we hypothesized that 3DG-collagen could be activating ASK1 while native collagen could promote the phosphorylation of p38 MAPK through the activation of HRAS. To test this, we cultured normal human dermal fibroblasts on native collagen or 3DG-collagen for 24 h, and performed Western blot analysis on the expression of HRAS and phospho-ASK1. Compared to fibroblasts cultured on native collagen, fibroblasts cultured on 3DG-collagen showed a 58% $\pm$ 12.4% decrease in the expression of HRAS, with a corresponding 90% $\pm$ 10.4% increase in the level of phospho-ASK1 (Fig. 38, $p < 0.002$). Additionally, we observed an increase in the level of phospho-p38 MAPK when cells were grown on 3DG-collagen ($p < 0.04$). These data suggest that fibroblasts cultured on native collagen most likely signal through the growth kinase HRAS, while fibroblasts cultured on 3DG-collagen signal through the stress kinase ASK1.

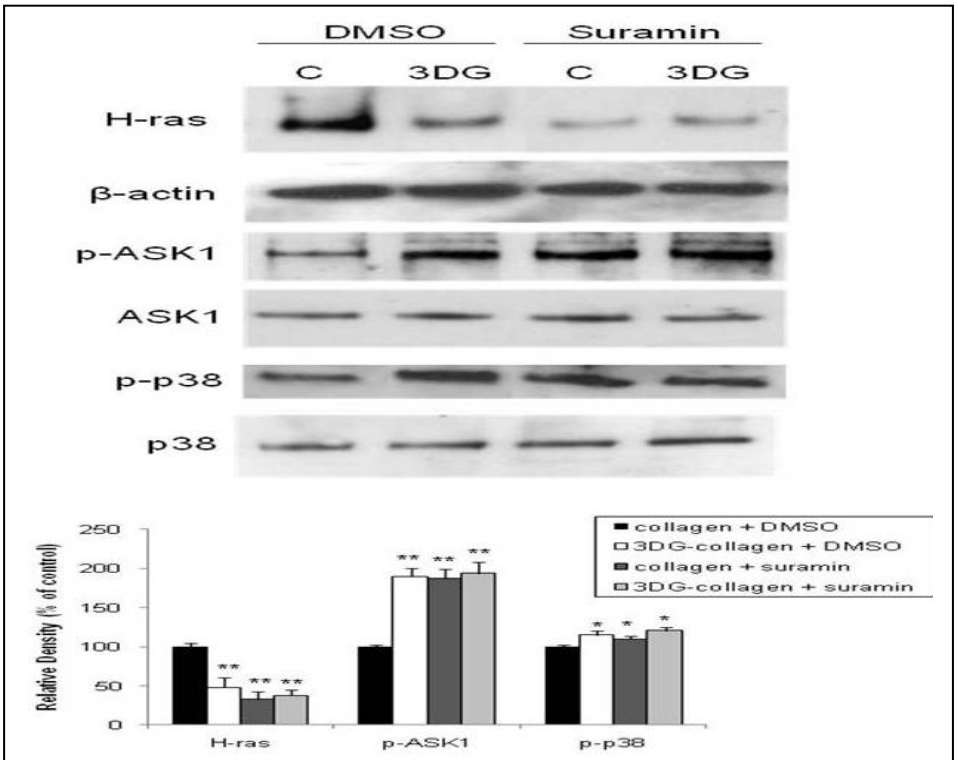

Figure 38. 3DG-collagen and suramin differentially regulate HRAS and phospho-ASK1 expression. Dermal fibroblasts that were 70% confluent were pretreated with suramin or vehicle DMSO for 1 h and cultured on native collagen or 3DG-collagen for 24 h. Using whole cell lysates, expression levels of phospho-p38 MAPK, phospho-ASK1 and HRAS were analyzed by Western blot. Total p38 MAPK, total ASK1, and β-actin served as loading controls. Results were quantified by densitometric scanning of the Western blot and phospho-p38 MAPK was normalized to total p38 MAPK, phospho-ASK1 was normalized to total ASK1, and HRAS was normalized to β-actin. All statistical comparisons are made against native collagen treated with DMSO. Data are mean ± SD (n=3), ****P<0.002, *P<0.04.**

Growth factors are responsible for activating HRAS signaling. Therefore, to determine if blockade of growth factor signaling could alter the expression of HRAS and ASK1 in fibroblasts cultured on native collagen and 3DG-collagen, we employed the use of suramin, a growth factor receptor inhibitor. Fibroblasts pretreated with suramin and cultured on native collagen resulted in a 70%± 10% decrease in the expression of HRAS and an 88% ± 10.8% increase in the phosphorylation of ASK1 (Fig. 38, $p < 0.002$). We observed no significant difference in the expression of HRAS or ASK1 between fibroblasts pretreated with suramin and then cultured on 3DG-collagen and fibroblasts cultured on 3DG-collagen alone. These data suggest that the inactivation of growth factor receptors in fibroblasts cultured on native collagen causes HRAS expression to be depressed resulting in the upregulation of ASK1 phosphorylation. Additionally, this response was similar to that observed in fibroblasts cultured on 3DG-collagen.

p38 MAPK differentially regulates the phosphorylation of ERK1/2 and AKT. Previous studies have shown that p38 MAPK can cross-talk with other pro-survival kinases, including ERK1/2 and AKT (42). During cell proliferation and migration, p38 MAPK, ERK1/2, and AKT are simultaneously upregulated (43). However, during cellular stress, p38 MAPK can inhibit the phosphorylation of ERK1/2 and AKT resulting in decreased proliferation and migration of the cell and increased apoptosis (42). We have previously shown that 3DG-collagen can downregulate the phosphorylation of ERK1/2 (33); therefore, we investigated if 3DG-collagen-induced p38 MAPK signaling modulated the phosphorylation of ERK1/2 and AKT. Fibroblasts were pretreated with SB202190 (p38 MAPK inhibitor) or the vehicle DMSO for 1 h and then cultured on native collagen or 3DG-collagen for 24 h. Phosphorylation of ERK1/2 and AKT was detected by Western blotting. Inhibition of p38 MAPK with SB202190 in fibroblasts cultured on 3DG-collagen increased the expression of phospho-ERK1/2 from 38% ± 7.8% to that observed in fibroblasts cultured on native collagen 95% ± 6.2% (Fig. 39, $p < 0.0001$) . Likewise we observed that phospho-AKT increased from 48% ± 4.4% in cells cultured on 3DG-collagen to 91% ± 2.4% in fibroblasts cultured on 3DG-collagen with the inhibitor SB202190 (Fig. 39, $p < 0.0001$). However, in fibroblasts cultured on native collagen, we observed that inhibition of p38 MAPK down regulated phospho-ERK1/2 to 43% ± 8.9% and phospho-AKT to 35% ± 4.8% (Fig. 39, $p < 0.0001$) suggesting 3DG changes the crosstalk between p38 MAPK, ERK1/2, and AKT. Pretreatment with suramin reduced the phospho-ERK1/2 and phospho-AKT expression to 37% ± 6.6% and 46% ± 8.8%, respectively; in fibroblasts cultured on native collagen (Fig. 39, $p < 0.0001$). Moreover, we found that this down regulation of phospho-ERK1/2 and phospho-AKT was dependent on the activation of p38 MAPK as inhibition of this kinase restored the phosphorylation of ERK1/2 and AKT to that observed in control cells (Fig. 39). These results suggest that

p38 MAPK in the presence of native collagen can act as a growth kinase promoting the phosphorylation of both ERK1/2 and AKT. However, the signaling from p38 MAPK in fibroblasts cultured on 3DG-collagen or in fibroblasts treated with the growth factor receptor inhibitor suramin, altered p38 MAPK to act as a stress kinase resulting in the depression of ERK1/2 and AKT phosphorylation. This data was published in Plos One, 2011.

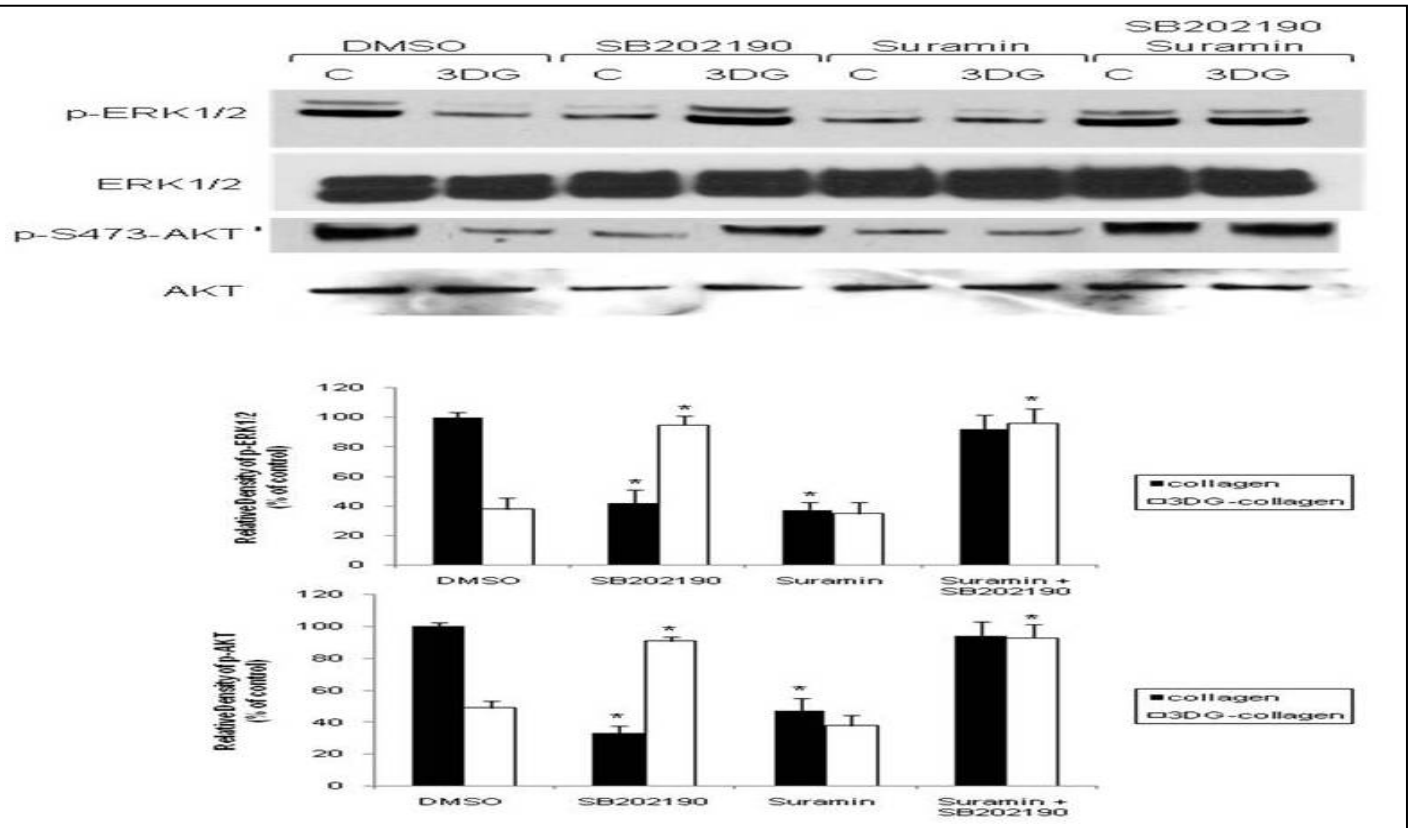

Figure 39. Expression of ERK1/2 and AKT after pretreatment with suramin and p38 inhibitor SB202190. 70% confluent fibroblasts were pretreated with suramin, SB202190, or vehicle DMSO for 1 h and cultured on native collagen or 3DG-collagen for 24 h. Expression of phopsho-ERK1/2 and phospho-AKT were analyzed by Western blot using whole cell lysates. Total ERK1/2 and total AKT served as loading controls. The bars correspond to the densitometric values of phospho-ERK1/2 and phospho-AKT after normalization for total ERK1/2 and total AKT, respectively. All comparisons are made against their respective controls corresponding to native collagen treated with DMSO or 3DG-collagen treated with DMSO. Data are mean $\pm$ SD (n=3), ***P < 0.0001**.

p38 MAPK inversely regulates the migration of fibroblasts cultured on 3DG-collagen or native collagen or when treated with suramin. It was previously demonstrated that 3DG-collagen down regulated the migration of dermal fibroblasts in an *in vitro* wound site (33). Since we observed that the inhibition of p38 MAPK in fibroblasts cultured on 3DG-collagen restored the level of phospho-ERK1/2 and phospho-AKT to levels observed in fibroblasts cultured on native collagen, and the phosphorylation of these proteins are known to promote the growth and migration of fibroblasts; we sought to evaluate the significance of p38 MAPK on fibroblast migration. Utilizing an *in vitro* scratch assay, fibroblasts were pretreated with DMSO, suramin, SB202190 (p38 MAPK inhibitor), PD98059 (ERK1/2 inhibitor), or LY294002 (AKT inhibitor) for 1 h and then cultured on native collagen or 3DG-collagen until confluent. A scratch was made along the monolayer of cells and cells were cultured with the inhibitors for an additional 24 h or 48 h. Fibroblasts cultured on native collagen in the absence of any inhibitor had closed the wound by 95% $\pm$ 1.4% by 48 h, while fibroblasts cultured on 3DG-collagen had closed the wound by 68% $\pm$ 2.6% (Fig. 40A, $p < 0.0001$). In the presence of the p38 MAPK inhibitor SB202190, fibroblasts cultured on native collagen were unable to migrate efficiently into the wound, resulting in only 67%$\pm$ 3.3% closure by 48 h (Fig. 40A, $p < 0.0001$). However, inhibition of p38 MAPK in fibroblasts cultured on 3DG-collagen restored the migration of fibroblasts, closing the wound by 92% $\pm$ 2.2% in 48 h (Fig. 40A, $p < 0.001$). Pretreatment of the fibroblasts with the growth factor inhibitor suramin reduced the migration of the fibroblasts cultured on both native collagen and 3DG-collagen resulting in a 64% $\pm$

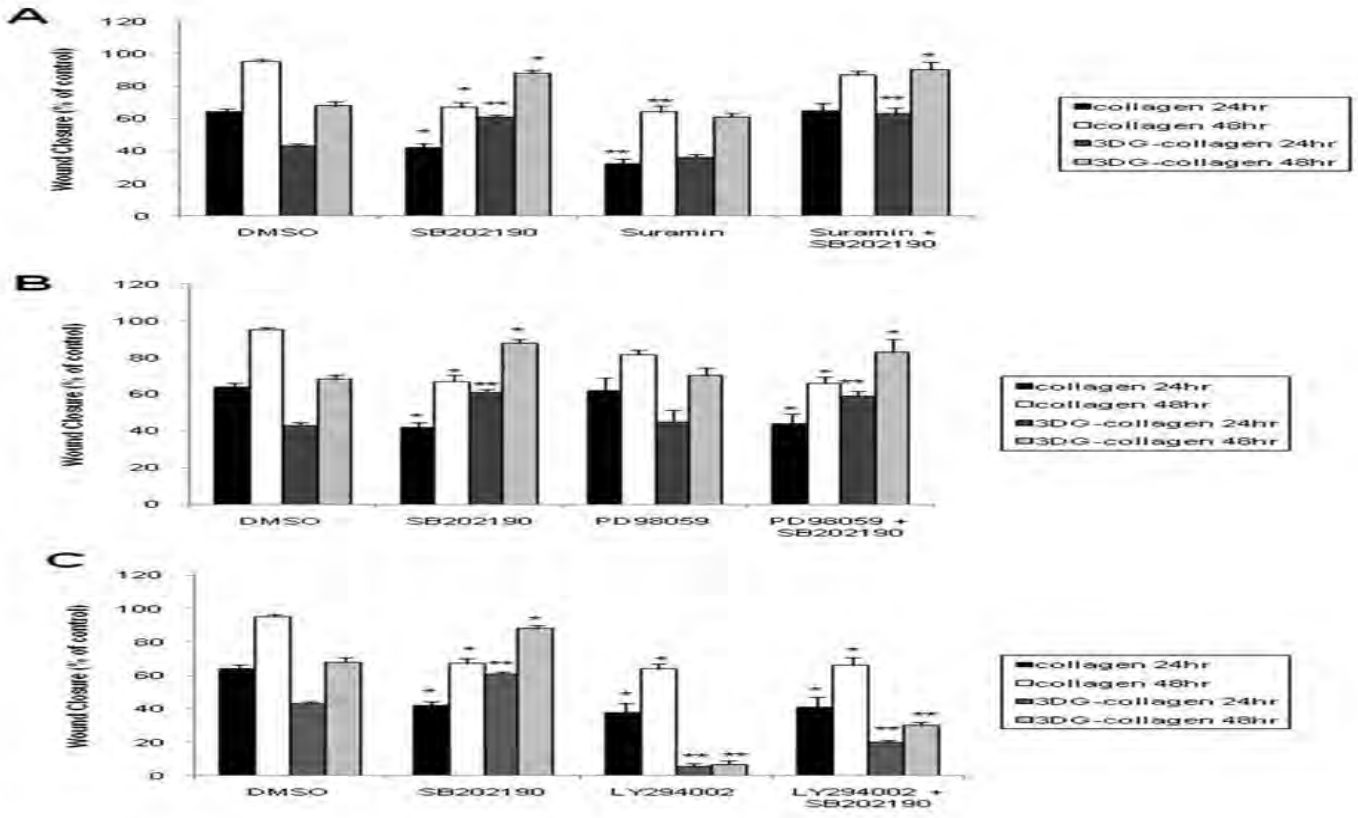

Figure 40. Wound closure rates in fibroblasts pretreated with suramin and kinase inhibitors. Confluent fibroblasts were pretreated with the inhibitors; growth factor inhibitor suramin, p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, or the ERK1/2 inhibitor PD98059 for 1 h and cultured on native collagen or 3DG-collagen. Cell migration into the wound was monitored at 0 h, 24 h, and 48 h by bright field visualization on an epi-fluorescence microscope. The distance across the wound margin was measured at 10 different points using Spot software and plotted as percentage of wound closure when compared to initial scratch at 0 h. **A**, Wound closure rates of fibroblasts pretreated with suramin and SB202190 and cultured on native collagen and 3DG-collagen. **B**, Wound closure rates of fibroblasts pretreated with SB202190 and PD98059 when cultured on native collagen or 3DG-collagen. **C**, Wound closure rates of fibroblasts pretreated with SB202190 and LY294002 when cultured on native collagen or 3DG-collagen. Statistical comparisons were performed within each time point and compared to their respective controls (native collagen or 3DG-collagen). Data are mean $\pm$ SD (n=3), ****P< 0.0001, *P < 0.001.**

3.6% and 61% $\pm$ 2.4% closure of the wound by 48 h, respectively (Fig. 40A, $p < 0.0001$). In addition, inhibition of p38 MAPK restored the migration of fibroblasts pretreated with suramin to that observed in the control. These results suggest that p38 MAPK may be acting as a stress kinase in the presence of 3DG-collagen and/or suramin resulting in decreased migration, while p38 MAPK may act as a growth response kinase in fibroblasts cultured on native collagen allowing for fibroblast migration and closure of the wound (Fig. 40A, $p < 0.001$). Moreover, the down regulation of HRAS by suramin and 3DG-collagen that we observed in Fig. 38 may be contributing to the altered signaling properties of p38 MAPK resulting in decreased cell migration. This data was published in Plos One, 2011.

To investigate further how p38 MAPK may be altering cellular migration we investigated the growth kinases ERK1/2 and AKT, which are known to regulate wound closure. Fibroblasts pretreated with the ERK1/2 inhibitor PD98059 and cultured on either native collagen or 3DG-collagen did not alter the rate of wound closure compared to that seen in their respective control groups (Fig. 40B). Moreover, inhibition of both ERK1/2 and p38 MAPK resulted in wound closure rates similar to that seen in fibroblasts pretreated with the p38 MAPK inhibitor alone. These results suggest that regulation of wound closure by p38 MAPK is not dependent on ERK1/2 activation. Pretreatment of fibroblasts cultured on native collagen with the AKT inhibitor LY294002, closed the wound by only 64% $\pm$ 2.8% in 48 h ($p < 0.001$), and the addition of LY294002 to fibroblasts cultured on 3DG-collagen resulted in a significant reduction in cell migration, as only 6.7% $\pm$ 2.1% of the wound was closed in 48 h (Fig. 40C, $p < 0.0001$). The addition of the p38 MAPK inhibitor SB202190 did not further alter the wound closure rates of fibroblasts cultured on native collagen (66% $\pm$ 4.8%)

or 3DG-collagen (30% ± 2.2%) suggesting that p38 MAPK regulation of wound closure is dependent upon the activation of AKT (Fig. 40C).

One of the main migratory features of dermal fibroblasts is the extension of their filopodia along the collagen matrix (44). After mechanical wounding, fibroblasts begin to extend their filopodia into the wound site by 4 h (33). Therefore, we investigated the effect of p38 MAPK on filopodia extension of fibroblasts cultured on native collagen and 3DG-collagen after mechanical wounding. Fibroblasts were pretreated with either the p38 MAPK inhibitor SB202190 or the growth factor receptor inhibitor suramin and the cultured on native collagen or 3DG-collagen until confluent. After confluency, a scratch was made and the actin filaments were stained using rhodamine phalloidin at 4 h post-scratch. As seen previously, fibroblasts cultured on native collagen increased their filopodia by 4 h. In contrast, fibroblasts grown on 3DG-collagen showed minimal extension of their filopodia at 4 h (Fig. 41). Inhibition of p38 MAPK with SB202190 delayed filopodia extension into the wound site when fibroblasts were cultured on native collagen. However, inhibition of p38

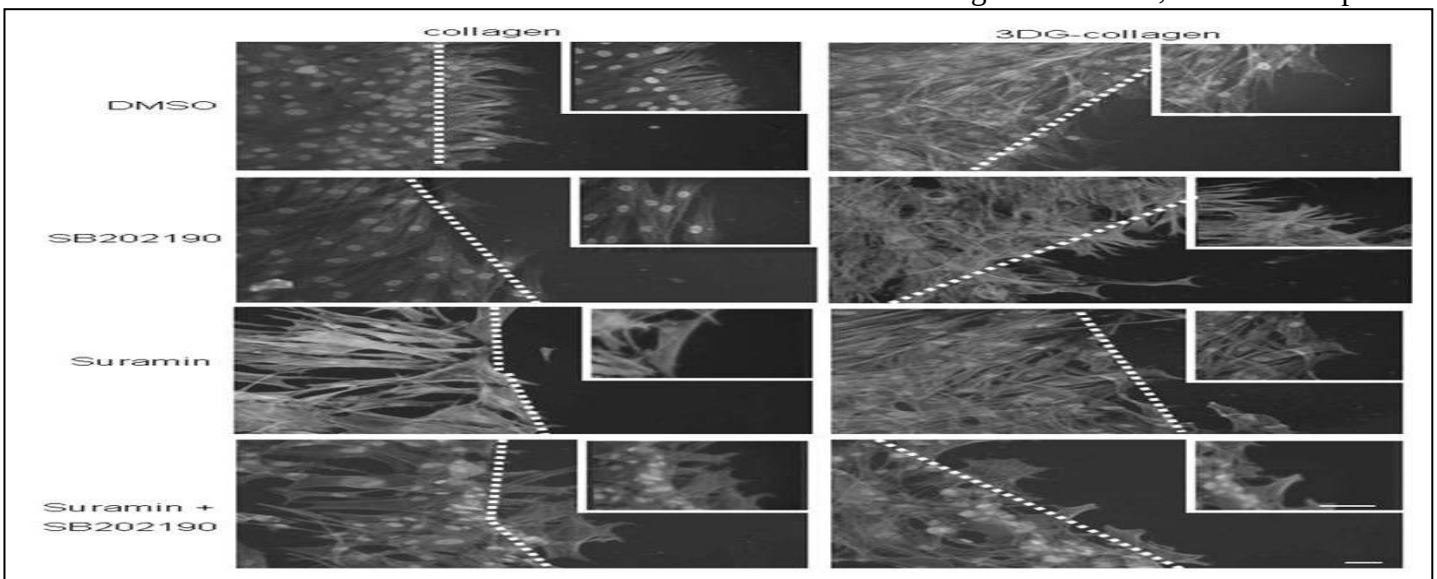

Figure 41. Differential regulation of filopodia extension after fibroblast pretreatment with suramin and p38 MAPK inhibitor SB202190. Fibroblasts were pretreated for 1 h with the inhibitors; growth factor receptor inhibitor suramin, the p38 MAPK inhibitor SB202190, or the vehicle DMSO and then cultured on native collagen or 3DG-collagen until confluent. The monolayer of cells was then manually scratched with a pipette tip to introduce the wound. At 4 h post-scratch the fibroblasts were fixed, permeabilized with Triton X-100, and stained with the F-actin dye rhodamine phalloidin. Extension was denoted as filopodia protrusion from initial wound site. The dotted line denotes initial wound site taken at 20 X magnification. Inset picture taken at 40 X magnification. Scale bar represents 10 μm. 10 images of each sample were taken at 20 X and 40 X magnifications, on an epi-fluorescence microscope with an exposure time of 2.2 s. Images represent 1 of 3 independent experiments.

MAPK induced filopodia extension in fibroblasts cultured on 3DG-collagen (Fig. 41). Additionally, suramin reduced the filopodia extension of fibroblasts cultured on native collagen to that seen in 3DG-collagen. Inhibition of p38 MAPK restored the filopodia extension of fibroblasts pretreated with suramin and then cultured on native collagen or 3DG-collagen to that seen in the control (Fig. 41). These results suggest that when fibroblasts are cultured on native collagen HRAS dependent activation of p38 MAPK is required for proper filopodia extension, while decreased HRAS may result in ASK1 upregulation of p38 MAPK in fibroblasts grown on 3DG-collagen leading to inhibition of filopodia extension. These results corroborate the current findings that p38 MAPK differentially regulates cellular migration, which is dependent upon the dermal fibroblast's interaction with extracellular stimuli. Moreover, these results are consistent with the findings that p38 MAPK reduces cell migration through down regulation of the phosphorylation of AKT when fibroblasts are cultured on 3DG-collagen as this kinase is needed for proper migration and cell survival.

Fibroblast proliferation is dependent upon p38 MAPK activation of AKT. Previous data from our laboratory has shown that 3DG-collagen significantly reduces fibroblast proliferation by 24 h (33;45). Both ERK1/2 and AKT are known kinases integrally involved in cell proliferation (42). Because p38 MAPK can inversely regulate the expression of these kinases in both a stress and growth environment, we investigated the role of p38 MAPK in cell proliferation. Fibroblasts were pretreated with suramin, SB202190, LY294002,

PD98059, or a combination of the inhibitors and cultured on either native collagen or 3DG-collagen. Cell proliferation was measured at 0 h, 24 h, and 48 h. Fibroblasts cultured on native collagen steadily proliferate over 48 h, while fibroblasts cultured on 3DG-collagen show a decrease in their proliferative capacity within 48 h (Fig. 42A, $p < 0.001$). There was a down regulation in the rate of proliferation when p38 MAPK was inhibited in fibroblasts cultured on native collagen compared to control. When p38 MAPK was inhibited with SB202190 in fibroblasts cultured on 3DG-collagen, proliferation was restored. These fibroblasts had proliferated to similar numbers at 48 h to that seen in fibroblasts cultured on native collagen. Pretreatment with suramin reduced the fibroblast's ability to proliferate on native collagen similar to that seen in fibroblasts cultured on 3DG-collagen. Furthermore, with the addition of the p38 MAPK inhibitor SB202190 to fibroblasts pretreated with suramin, the proliferation rate was restored to that observed in fibroblasts cultured on native collagen suggesting that down regulation of HRAS may alter the phenotype of p38 MAPK (Fig. 42A, $p < 0.03$).

Next we investigated what kinase p38 MAPK was regulating to induce proliferation. Fibroblasts were pretreated with the ERK1/2 inhibitor PD98059 and the AKT inhibitor LY294002 and proliferation was measured over 48 h. We found that the rate of proliferation in fibroblasts cultured on native collagen was

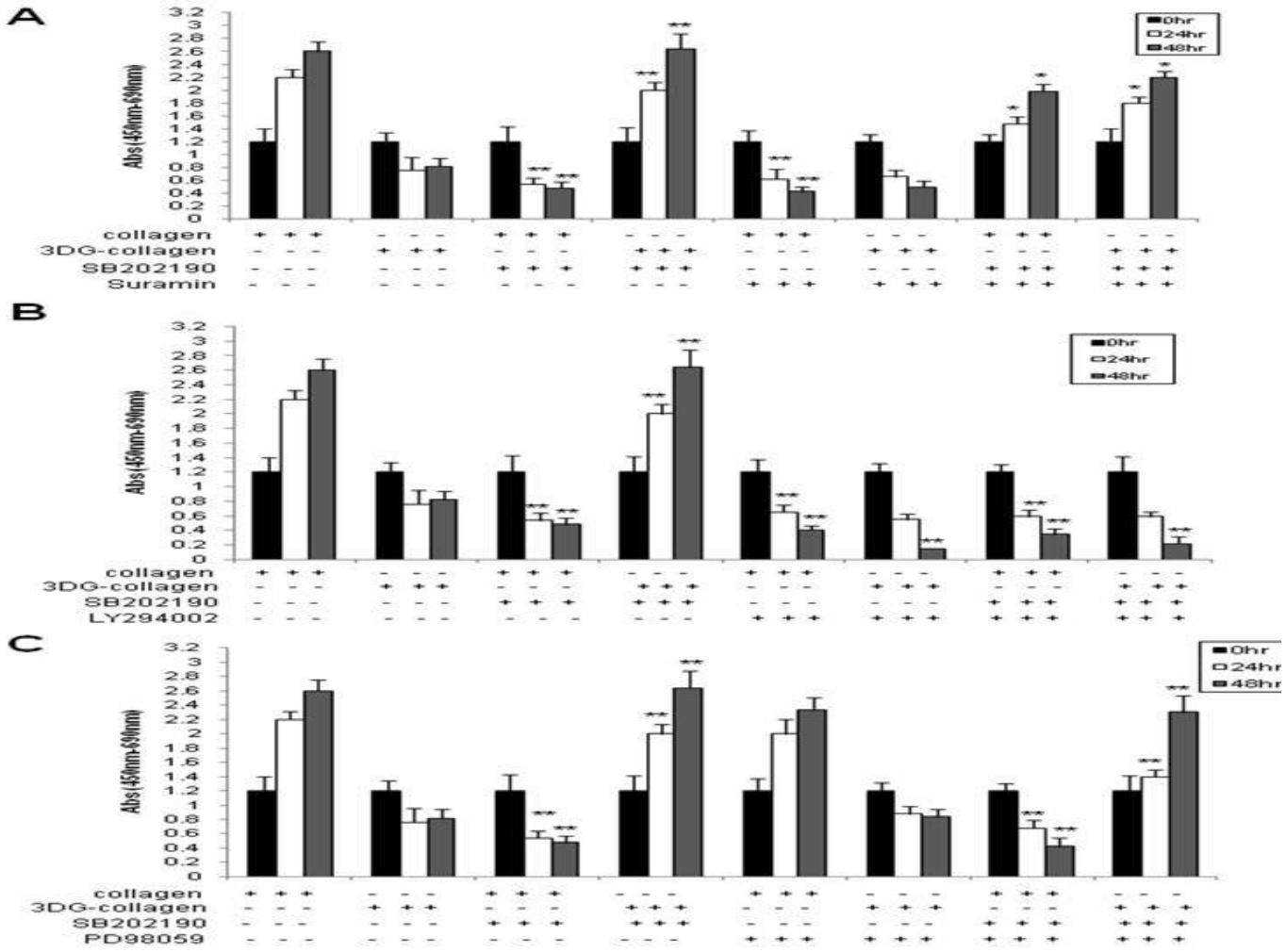

Figure 42. Proliferation rates in fibroblasts pretreated with suramin and kinase inhibitors. 4 X 10^3 cells/well were pretreated with the growth factor receptor inhibitor suramin, p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, or ERK1/2 inhibitor PD98059 for 1 h and then seeded in a 96-well plate containing either native collagen or 3DG-collagen for 0 h, 24 h, and 48 h. At each designated time point the level of proliferation was determined using the cell proliferation reagent WST-1. Quantification of each sample was determined by measuring the absorbance at 450 nm, with a reference wavelength of 690 nm. **A**, Proliferation rates of fibroblasts pretreated with suramin and SB202190 and cultured on native collagen or 3DG-collagen. **B**, Proliferation rates of fibroblasts pretreated with SB202190 and LY294002 and cultured on native collagen or 3DG-collagen. **C**, Proliferation rates of fibroblasts pretreated with SB202190 and PD98059 and cultured on native collagen or 3DG-collagen. Comparisons were performed within each time point and compared to their respective controls (native collagen or 3DG-collagen). Data are mean $\pm$ SD (n=3), ****P < 0.001, *P < 0.03.**

dependent on AKT but not ERK1/2 as only inhibition of AKT showed similar levels of proliferation to that seen in fibroblasts pretreated with the p38 MAPK inhibitor (Fig. 42B, C; $p<0.001$). Moreover, the simultaneous inhibition of p38 MAPK and AKT did not provide any significant additive effect to the decreased rate of proliferation seen in these fibroblasts, suggesting that proliferation is dependent on the p38 MAPK regulation of AKT (Fig. 42B, $p<0.001$). In addition, fibroblasts pretreated with AKT alone and cultured on 3DG-collagen showed no signs of proliferation in the first 24 h and by 48 h the majority of the cells had died, while there was no change in the proliferation capacity of these cells when ERK1/2 was inhibited (Fig. 42C; $p<0.001$). These studies suggest that 3DG-collagen-induced p38 MAPK downregulates cell proliferation by down regulating the phosphorylation of AKT.

Upregulation of the collagen receptor α1β1 integrin by 3DG-collagen and suramin is dependent on growth factor signaling and H-ras expression. H-ras has been shown to regulate integrin activation through downregulation of integrin transcription and the binding of fibroblasts to collagen is mediated through two integrins receptors; α1β1 integrin and α2β1 integrin. Since 3DG altered the adhesion of fibroblasts to collagen, we examined the expression of these collagen receptors when fibroblasts were cultured on native collagen, 3DG-collagen, or treated with the growth factor inhibitor suramin. Quantitative real-time PCR showed that fibroblasts cultured on 3DG-collagen increased their expression of β1 integrin and α1 integrin to 188% $\pm$ 12.7% and 162% $\pm$ 8.8%, respectively (Fig. 43A; $p<0.001$). The expression of α2 integrin only slightly increased to 115% $\pm$ 11.8% as compared with fibroblasts cultured on native collagen (Fig. 43A). Additionally, fibroblasts pretreated with suramin and cultured on native collagen showed increased expression of β1 integrin and α1 integrin but not α2 integrin; 175% $\pm$ 12%, 192% $\pm$ 14.2%, and 99% $\pm$ 14.3%, respectively (Fig. 43A; $p<0.001$). Pretreatment of fibroblasts cultured on 3DG-collagen with suramin did not significantly enhance the level of integrin expression compared to fibroblasts cultured on 3DG-collagen alone (Fig. 43A).

In addition to increased integrin transcript levels, we also investigated the protein expression of these integrins in fibroblasts cultured on native collagen, 3DG-collagen, and treated with suramin. Fibroblasts cultured on 3DG-collagen increased the expression of both β1 integrin and α1 integrin to 165% $\pm$ 2.2% and 148% $\pm$ 16%, respectively (Fig. 43B; $p<0.001$ and $p<0.01$). As seen with the transcript levels there was no significant increase in the expression of α2 integrin in fibroblasts cultured on 3DG-collagen compared to those cultured on native collagen. Moreover, fibroblasts pretreated with suramin and cultured on native collagen increased their expression of β1 integrin and α1 integrin to 168% $\pm$ 28% and 141% $\pm$ 4.5%, respectively (Fig. 43B; $p<0.01$ and $p<0.001$). The increase in protein expression of β1 integrin and α1 integrin correlated with the downregulation of H-ras by 3DG-collagen and suramin (Fig. 43B). Taken together, these data suggests that downregulation of H-ras by 3DG-collagen or suramin increases the expression of the collagen receptor α1β1 integrin at both the level of transcription and translation.

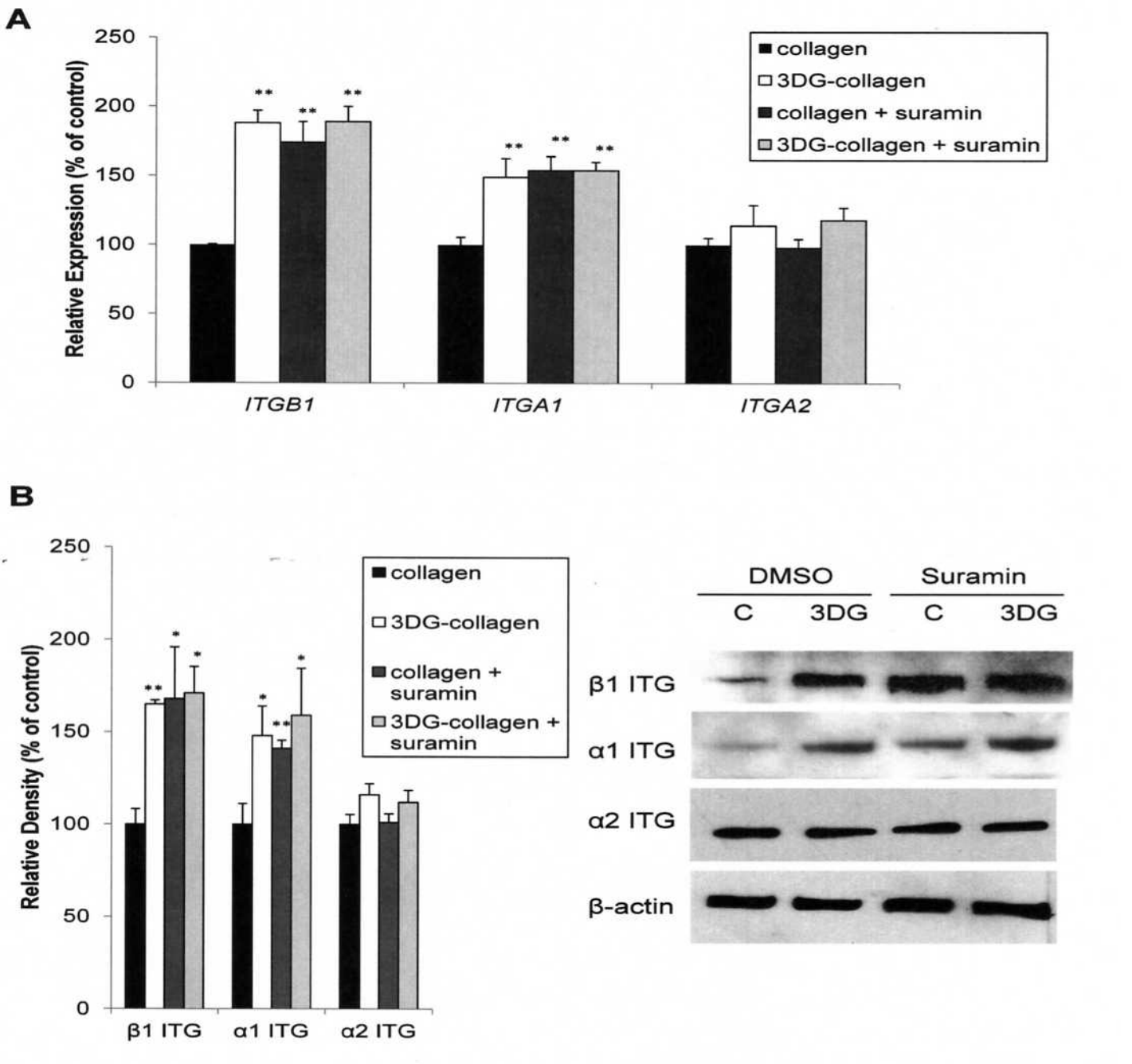

Figure 43. 3DG-collagen and suramin upregulates the expression of α1β1 integrin. Fibroblasts were pretreated with suramin or vehicle DMSO for 1 h and cultured on either native collagen or 1mM 3DG-collagen for 24 h. **A**, mRNA was analyzed for the expression of β1, α1, and α2 integrin by real-time PCR. All transcripts were normalized to β-actin. **B**, Fibroblasts were treated as in A and analyzed for the expression of β1, α1, and α2 integrin by Western blot. The bars correspond to the densitometric value of β1, α1, and α2 integrin after normalization for β-actin. All comparisons are made against native collagen treated with PBS. Data are mean ± SD (n=3), **$P < 0.001$, *$P < 0.01$.

Growth factor expression is dependent on α1β1 integrin upregulation by 3DG-collagen. H-ras expression is controlled by the activation of growth factors. 3DG-collagen reduces the expression of both growth factor receptors and their respective ligands (Fig. 44). Therefore we examined the role of integrins in controlling the expression of growth factors that in turn should control the activation of H-ras. Fibroblasts were preincubated with blocking mAbs targeting β1, α1, or α2 integrin, and then cultured on native collagen or 3DG-collagen for 24 h. The integrin subunits were only partially blocked as full blockade of these subunits resulted in cell detachment from the collagen coating and anoikis (data not shown). Quantitative real-time PCR revealed that blockade of β1 and α1 integrin but not α2 integrin reversed the 3DG-collagen induced downregulation of all

growth factors and their receptors (Fig. 44A-C, $p<0.05$). Furthermore, preliminary results are beginning to reveal that blockade of β1 and α1 integrin abrogates the reduction in H-ras expression when fibroblasts are cultured on 3DG-collagen (data not shown). These results suggest that α1β1 integrin interacts with 3DG-collagen resulting in a downregulation of growth factor receptors and their ligands, which may result in a decrease in H-ras expression.

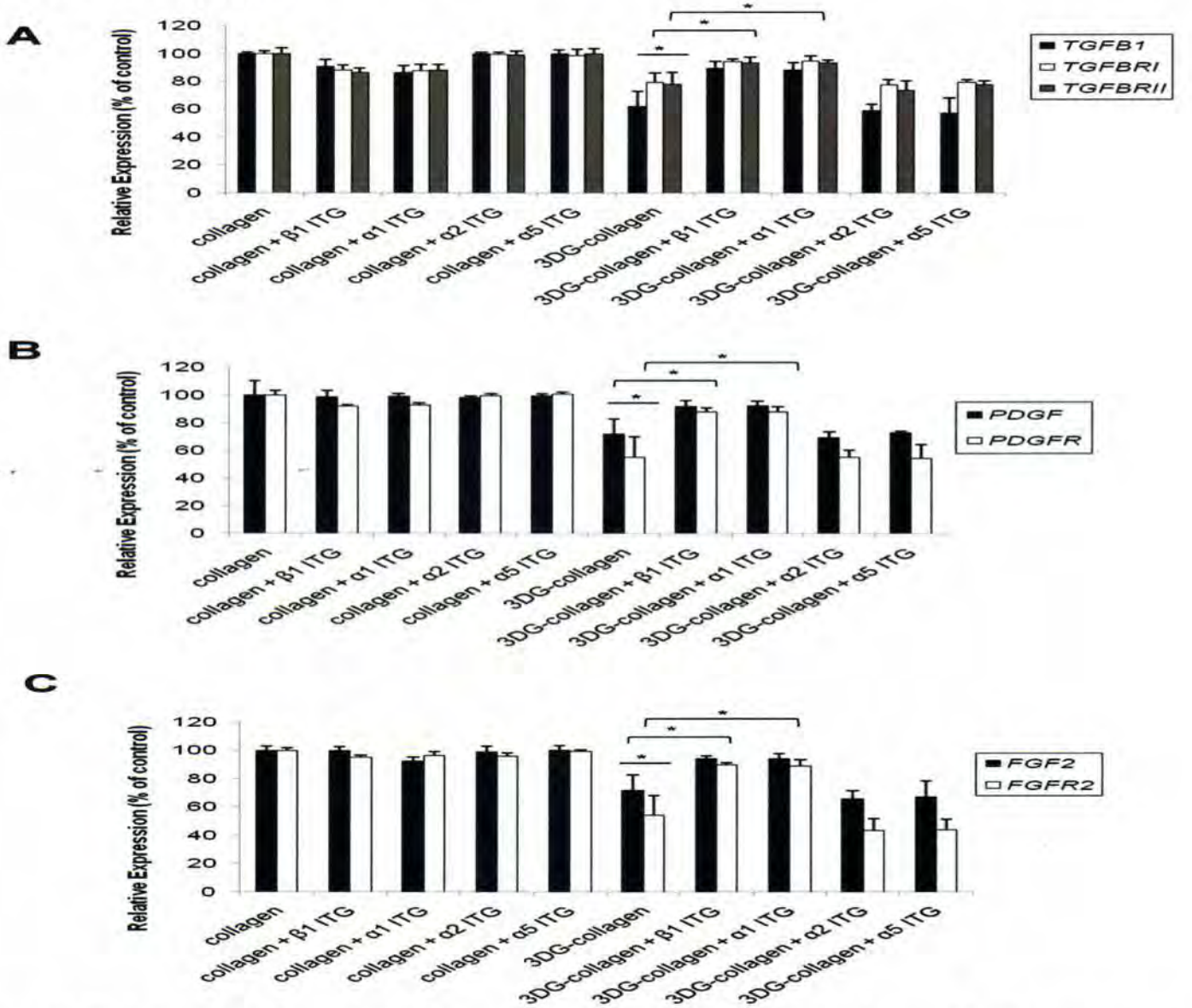

Figure 44. Blockade of α1β1 abrogates 3DG-collagen induced downregulation of growth factors. Fibroblasts were pretreated with or without the blocking antibodies anti-β1 ITG, anti-α1 ITG, anti-α2 ITG, and anti-α5 ITG (1:50 dilution) for 30 min and cultured on native collagen or 1mM 3DG-collagen for 24 h. **A**, TGFβ1, TGFβRI, TGFβRII, **B**, PDGF, PDGFR, **C**, FGF-2, and FGFR-2 mRNA expression levels were quantified by real-time RT-PCR. All transcripts were normalized to β-actin. All comparisons are made against native collagen. Data are mean + SD (n=3), ***P < 0.05.**

Reduced fibroblast migration on 3DG-collagen is dependent on α1β1 integrin. As shown in Fig. 41, fibroblasts cultured on 3DG-collagen (DMSO control) show reduced capacity to migrate into wounds. Moreover, 3DG-collagen depletes H-ras, which is necessary for proper cell spreading and migration along the ECM. In Fig. 22, α1β1 integrin was shown to adhere strongly to 3DG-collagen and this adhesion may result in reduced cell migration. Because inhibition of α1β1 integrin reversed the 3DG-collagen-induced inhibition of growth factors and H-ras expression, we focused our attention on the role of α1β1 integrin on cell migration into a wound site composed of 3DG-collagen. Fibroblasts were pretreated with blocking mAbs targeting β1, α1, or α5 integrin, and then cultured on native collagen or 3DG-collagen until confluent. Additionally, fibroblasts were pretreated with suramin to look at the rate of cell migration after depletion of growth factor signaling on

native collagen. When confluent, a "wound" was made across the monolayer of fibroblasts by a manual pipette tip and then washed to remove any non-adherent or damaged cells. Suramin and the blocking mAbs were added back to media containing 1% FBS-DMEM and the rate of wound closure was measure at 0 h, 24 h, and 48 h. As seen in Figure 40, fibroblasts cultured on 3DG-collagen showed a reduced capacity to close the wound by 48 h in comparison to fibroblasts cultured on native collagen. In addition, fibroblasts pretreated with suramin closed the wound by 64% $\pm$ 3.6% in 48 h, which was comparable to that seen in fibroblasts cultured on 3DG-collagen (Fig. 45, $p<0.05$). These results suggest that depletion of H-ras and growth factors modulates cell migration.

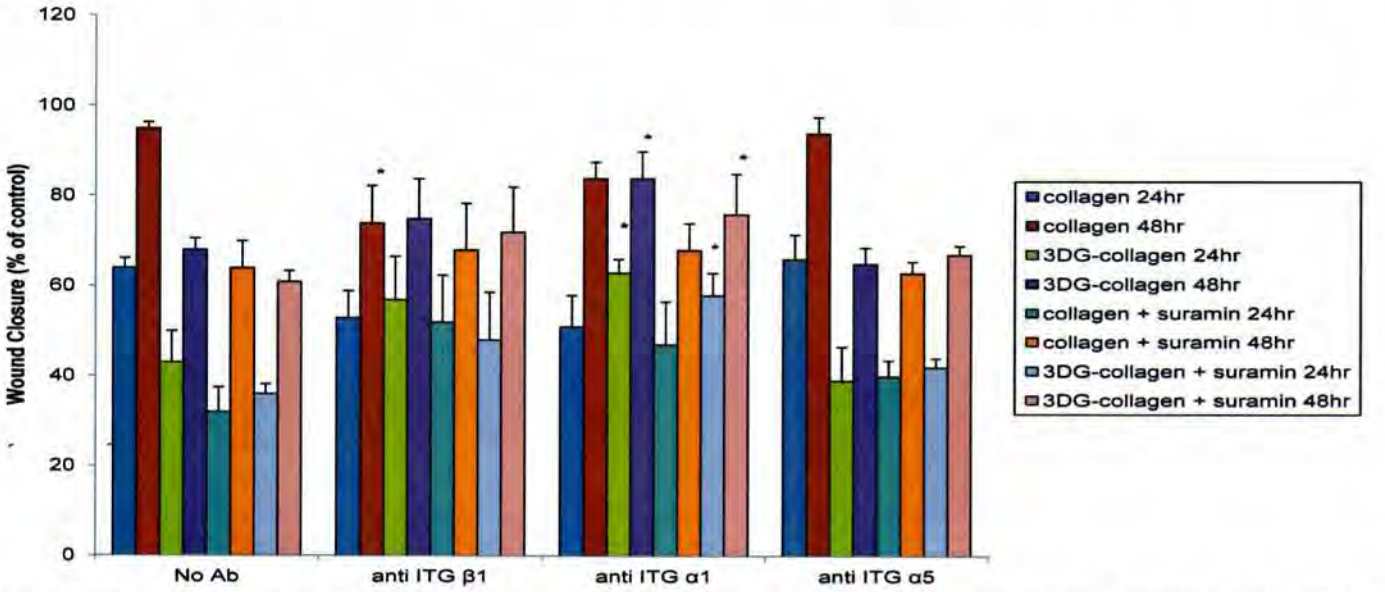

Figure 45. Fibroblast migration along 3DG-collagen is restored after α1β1 blockade. Fibroblasts were pretreated with suramin for 1 h and/or the blocking antibodies anti-β1 ITG, anti-α1 ITG, and anti-α5 ITG (1:50 dilution) for 30 min and cultured on native collagen or 1mM 3DG-collagen until confluent. The fibroblasts were then scratched manually with a pipette tip to introduce a wound. Cell migration into the wound was monitored at 0 h, 24 h, and 48 h by bright field visualization on an epi-fluorescence microscope. The distance across the wound margin was measured at 10 different and random points using Spot software and plotted as percentage of wound closure when compared to initial scratch at 0 h. Statistical comparisons were performed within each time point and compared to their respective controls (native collagen or 3DG-collagen). Data are mean $\pm$ SD (n=3), ***P < 0.05.**

Furthermore, blockade of α1 integrin resulted in a significant restoration of fibroblast migration along 3DG-collagen. Fibroblasts pretreated with blocking antibodies to α1 integrin promoted wound closure as 84% $\pm$ 6% of the wound was closed by 48 h (Fig. 45, $p<0.05$). Blockade of β1 integrin did not significantly increase fibroblast migration on 3DG-collagen. However , the lack of change could be due to the reduced availability of β1 integrin to bind to other α integrin subunits such as α2 integrin can also promote fibroblast migration. Blockade of the fibronectin receptor α5 integrin did not improve the rate of fibroblast migration along either native collagen or 3DG-collagen. However, blockade of α1 integrin in fibroblasts treated with suramin only partially closed the wound in fibroblasts cultured 3DG-collagen, while no change in migration was found in fibroblasts cultured on native collagen with suramin. These results suggest that fibroblast migration is also dependent on growth factor signaling. In support of this, blockade of β1 and α1 integrin in fibroblasts cultured on 3DG-collagen restored the growth factor receptor signaling (Fig. 44), however, in the presence of suramin growth factor signaling would still be inhibited despite the restored surface expression of growth factor receptors. These results suggest that the collagen receptor α1β1 integrin reduces fibroblast migration along 3DG-collagen, which results in reduced wound closure and this was partially mediated by growth factor signaling.

AKT activation is required for regulation of caspase-3 by p38 MAPK in fibroblasts cultured on native collagen and 3DG-collagen. ASK1 is known to activate p38 MAPK to be a pro-apoptotic kinase signaling the activation of apoptotic signaling cascades (37). However, recent studies have shown that HRAS-dependent upregulation of p38 MAPK can enhance cell survival in cancer cells through activation of AKT (38). 3DG-collagen has been shown to induce the expression of caspase-3 (33). Therefore, we investigated the role of p38

MAPK in caspase-3 activation under growth and stress conditions. When fibroblasts were pretreated with the p38 MAPK inhibitor SB202190 and cultured on native collagen, there was a 77% ± 5.2% increase in the level of caspase-3 activation (Fig. 46, $p < 0.001$). Moreover, when fibroblasts were pretreated with suramin, which is known to increase ASK1, and grown on native collagen there was an increase in caspase-3 activation to 285%± 10.2% and this was dependent on the activation of p38 MAPK (Fig. 46, $p < 0.0002$). To determine how p38 MAPK is regulating caspase-3 activation in fibroblasts cultured on native collagen we investigated the role of AKT and ERK1/2. The regulation of caspase-3 by p38 MAPK in fibroblasts cultured on native collagen was dependent on the activation of AKT as inhibition of AKT increased the level of caspase-3 to 189% ± 2.4% ($p < 0.001$). Additionally, there was no significant additive effect on the level of caspase-3 activation when both p38 MAPK and AKT were simultaneously inhibited (207% ± 3.2%). Moreover, the inhibition of ERK1/2 did not significantly increase the expression of caspase-3 (109% ± 3.3%), while inhibition of both ERK1/2 and p38 MAPK increased the expression of caspase-3 to 181% ± 4.6% suggesting that ERK1/2 is not responsible for the survival of the cell (Fig. 46, $p < 0.001$). Instead, survival of fibroblasts cultured on native collagen is dependent upon p38 MAPK-induced AKT activation.

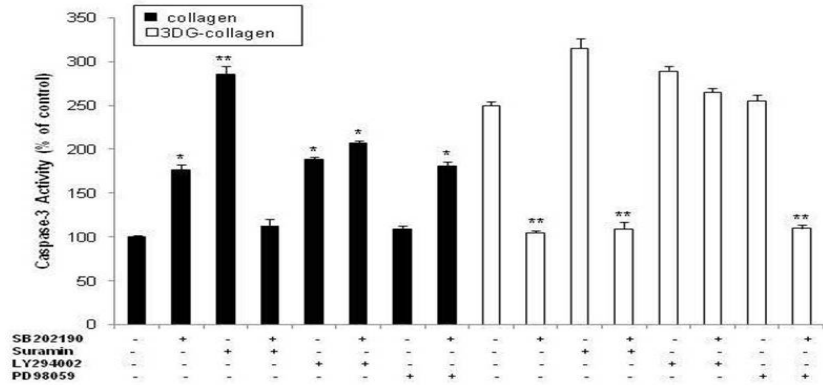

Figure 46. Measurement of caspase-3 activation after fibroblast pretreatment with suramin and kinase inhibitors. Fibroblasts were pretreated with the inhibitors; growth factor receptor inhibitor suramin, p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, or ERK1/2 inhibitor PD98059 for 1 h and then cultured on native collagen or 3DG-collagen for 24 h. Whole cell lysates were assayed for caspase-3 activity according to the protocol from Caspase-3 Colorimetric Correlate Assay. All samples were performed in triplicate and normalized to the control samples. All comparisons are made against their respective controls (native collagen or 3DG-collagen alone). Data are mean ± SD (n=3),
****P < 0.0002, *P < 0.001.**

We next investigated the role of p38 MAPK in fibroblasts cultured on 3DG-collagen. As seen previously, fibroblasts cultured on 3DG-collagen increased the expression of caspase-3 to 250%± 4.5% and this upregulation was abrogated when p38 MAPK was inhibited (Fig. 46, 105% ± 2.5%, $p < 0.0002$). Moreover, pretreatment of fibroblasts cultured on 3DG-collagen with suramin upregulated caspase-3 expression to that seen in fibroblasts cultured on 3DG-collagen alone, and this upregulation was dependent on p38 MAPK. These results suggest that suramin may be utilizing p38 MAPK in the same manner as 3DG-collagen to reduce fibroblast cell survival.

As shown previously p38 MAPK downregulates the phosphorylation of AKT (Fig. 39) and ERK1/2 (Fig. 39) in fibroblasts cultured on 3DG-collagen; therefore, we investigated whether down regulation of AKT or ERK1/2 by p38 MAPK was responsible for increased caspase-3 activation in fibroblasts cultured on 3DG-collagen. The activation of caspase-3 by p38 MAPK in fibroblasts cultured on 3DG-collagen is shown to be dependent on the inactivation of AKT. Inhibition of AKT caused a 189% ± 6.2% increase in the level of caspase-3 activation in fibroblasts cultured on 3DG-collagen (Fig. 46, $p < 0.0002$). This increase was not significantly altered when fibroblasts were pretreated with both AKT and p38 MAPK inhibitors simultaneously (165% ± 5.2%), unlike that seen in fibroblasts treated simultaneously with ERK1/2 and p38 MAPK inhibitors (155% ± 6.5% increase with ERK1/2 inhibitor vs. 10% ± 4.2% increase with ERK1/2 and p38 MAPK inhibitor). These results suggest that in fibroblasts cultured on 3DG-collagen p38 MAPK reduces the phosphorylation of AKT, which is responsible for increased caspase-3 activation.

Type I collagen expression is inversely regulated by p38 MAPK in fibroblasts cultured on native collagen and 3DG-collagen. During wound healing, type I collagen is synthesized by dermal fibroblasts to aid in successful closure of the wound margins (46). p38 MAPK has been shown to reduce collagen production in fibroblasts explanted from diabetic wounds (47) and we have demonstrated that 3DG-collagen inhibits the expression of type I collagen by the fibroblast (45, and Fig. 7); therefore, we investigated the role of p38 MAPK on type I collagen production. Fibroblasts were pretreated for 1 h with the inhibitors suramin, SB202190, LY294002, PD98059, or a combination; and cultured on native collagen or 3DG-collagen for 24 h. Fibroblasts cultured on native collagen induced the expression of collagen at both the level of transcription and translation. In contrast, fibroblasts cultured on 3DG-collagen reduced both the transcript levels of COL1A1 (75%$\pm$ 3.2%) and the protein levels of procollagen (62%$\pm$ 4.2%). Inhibition of p38 MAPK with SB202190 in fibroblasts cultured on native collagen showed both reduced transcript levels of COL1A1 (68%$\pm$ 3.2%, $p < 0.0001$) and reduced expression of procollagen (62%$\pm$ 6.5%, $p < 0.0001$). In contrast, inhibition of p38 MAPK restored the expression of COL1A1 (101%$\pm$ 4.2%) and procollagen (98%$\pm$ 4.4%) in fibroblasts grown on 3DG-collagen (Fig. 47A, B; $p < 0.0001$). Inhibition of growth factor receptors by suramin reduced the mRNA levels of COL1A1 to 71%$\pm$ 7.8% and the protein content of procollagen to 68%$\pm$ 7.2% in fibroblasts cultured on native collagen. This effect was similar to that observed in fibroblasts cultured on 3DG-collagen (Fig. 47A, B; $p < 0.0001$). Moreover, this effect was found to be dependent on the activation of p38 MAPK as inhibition of p38 MAPK in fibroblasts pretreated with suramin and cultured on native collagen abrogated the down regulation of COL1A1 (95%$\pm$ 9.8%, $p < 0.0001$) and procollagen (90%$\pm$ 10.0%, $p < 0.0001$).

p38 MAPK regulation of collagen is dependent on the activation of both AKT and ERK1/2 as there was a similar decrease in collagen expression when both p38 MAPK and AKT and p38 MAPK and ERK1/2 were

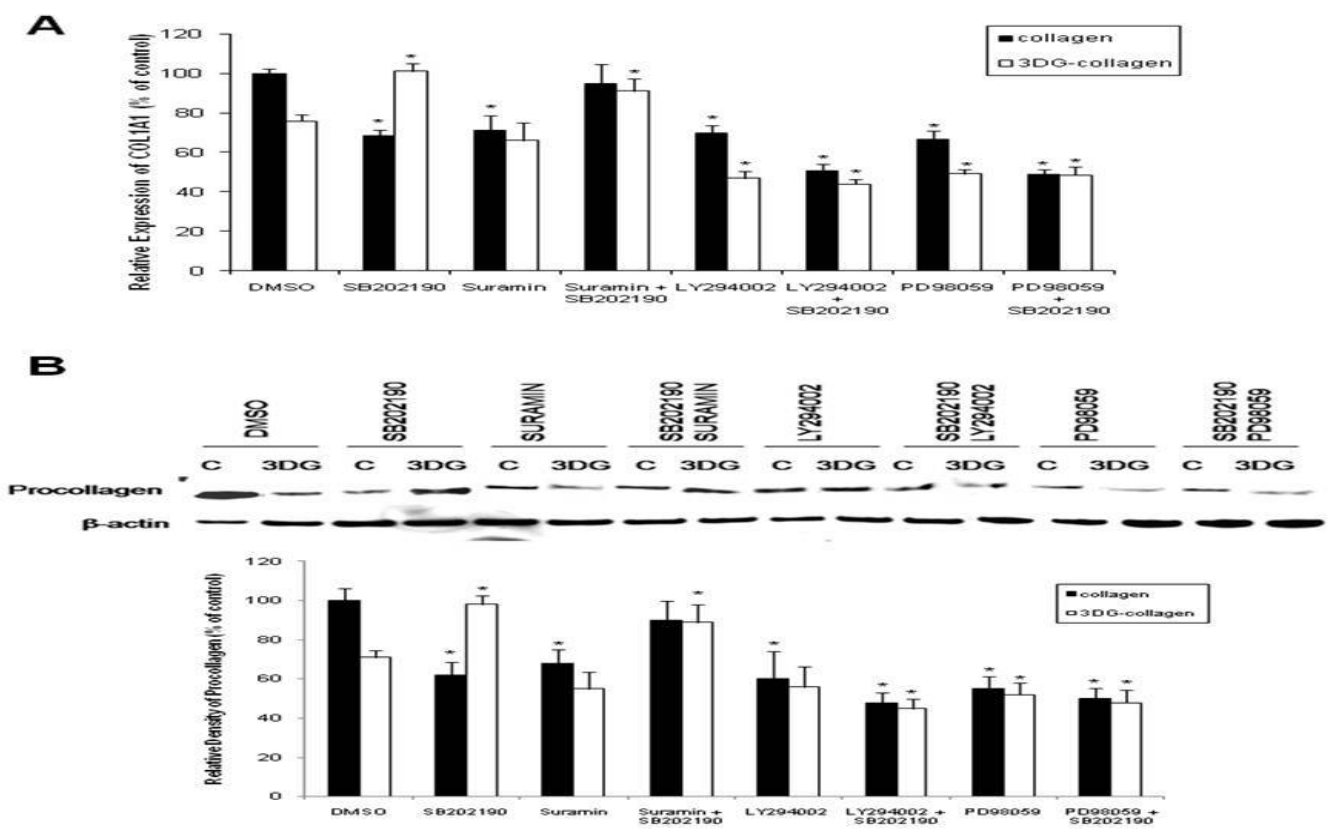

Figure 47. Expression of type I collagen after fibroblast pretreatment with suramin and kinase inhibitors. Fibroblasts were pretreated with the inhibitors; growth factor receptor inhibitor suramin, p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, or ERK1/2 inhibitor PD98059 for 1 h and then cultured on native collagen or 3DG-collagen for 24 h. **A**, COL1A1 mRNA expression levels were quantified by real-time RT-PCR. All transcripts were normalized to β-actin. **B**, Expression levels of procollagen were analyzed by Western blot and β-actin served as a loading control. The bars correspond to the densitometric value of procollagen after normalization for β-actin. All comparisons are made against their respective controls (native collagen or 3DG-collagen). Data are mean + SD (n=3), ***P < 0.0001.**

inhibited in fibroblasts grown on native collagen (Fig. 47A, B; p<0.0001). In fibroblasts cultured on 3DG-collagen, collagen expression was dependent on the p38 MAPK down regulation of both AKT and ERK1/2 as restoration of collagen expression by p38 MAPK inhibition was abrogated when both AKT and ERK1/2 were independently inhibited (Fig. 47A, B; p < 0.0001). These data suggest that p38 MAPK is playing a major role in the transcription and translation of collagen. p38 MAPK is playing a positive role in the regulation of collagen when fibroblasts are grown on native collagen, while it plays a negative role in fibroblasts grown on 3DG-collagen.

Specific Aim 3: Investigation of signaling in the ERK pathway of compounds that inhibit 3DG.

Modulation of Type I collagen expression by the signaling inhibitors, SB202190, LY294002, Ras, Atiprimod, or suramin in SSc Fibroblasts. Because 3DG-collagen induced a decrease in collagen expression in both normal and SSc fibroblasts and because we know the signaling pathways mediated by 3DG-collagen, we investigated the role of the inhibitors suramin (growth factor receptor), SB202190 (p38 MAPK inhibitor), LY294002 (AKT inhibitor), a Ras inhibitor, and Atiprimod a STAT3 inhibitor and determined collagen expression in SSc fibroblasts. We have made the following observations suggesting that these pathways are also operational in SSc fibroblasts and that inhibitors of these signaling molecules could be used to decrease collagen expression and therefore make the quality of life better in SSc patients. We found that the AKT inhibitor LY294002 worked slightly better than the p38 MAPK inhibitor SB202190.

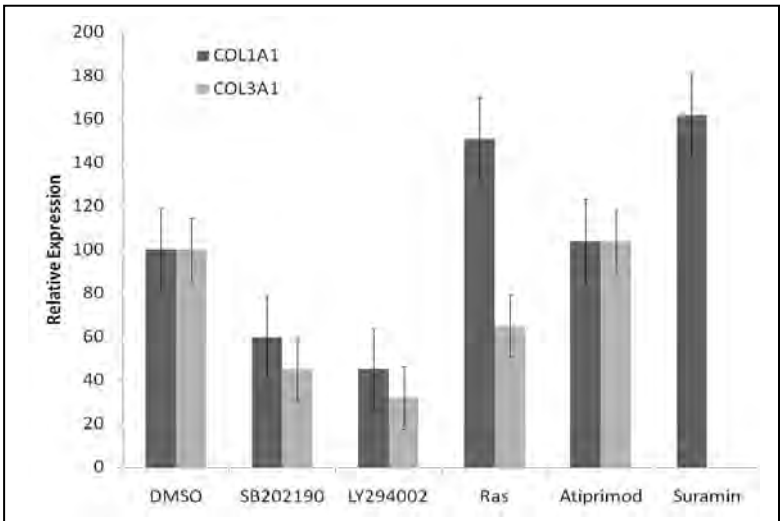

Figure 48. Collagen expression in SSc fibroblasts treated with SB202190, LY294002, Ras, Atiprimod, or Suramin. Confluent SSc fibroblasts were pretreated with the inhibitors; p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, Ras inhibitor, Atiprimod a STAT3 inhibitor, or Suramin a growth factor receptor inhibitor for 24 h. RNA was isolated and COL1A1 and COL3A1 transcripts measured by real-time PCR. DMSO was used as a control for the carrier solution. We found that SB202190 and LY294002 decreased collagen expression, whereas the Ras, Atiprimod, and suramin did not.

We further elucidated the signaling in SSc fibroblasts and determined that it is not operating through the Ras/MEK/ERK pathway. We found that when the Ras inhibitor or suramin was added to the SSc fibroblasts collagen expression was increased. This suggests that the signal is not coming from growth factor receptors but from the TGFβ1 receptor that directs signaling via AKT and p38. We have also investigated the inhibitor Atiprimod. This inhibitor targets STAT3 which receives its signal from JAK. JAK signaling can also affect AKT signaling. Indeed, on analysis of Atiprimod, we found collagen expression did not alter in SSc fibroblasts, further confirming the importance of the AKT pathway and that STAT3 did not contribute to SSc fibroblast signaling (Fig. 48).

Meglumine upregulates the expression of critical wound healing components. Meglumine is a compound that inhibits 3DG formation in vivo and in vitro, therefore we studied its affects on fibroblasts to determine if it would lower collagen. One of the characteristics of diabetic wounds is the reduction of critical ECM components which are necessary for proper wound contraction (48). Dermal fibroblasts are known to increase the production of type I collagen and α-smooth muscle actin (α-SMA), which allow for proper wound contraction and remodeling of the ECM (49). Furthermore, fibroblasts produce growth factors that are essential for infiltration and proliferation of fibroblasts into the wound site (50-52). Therefore, we investigated the effect of meglumine on the basal transcript levels of COL1A1, α-SMA, TGF-β1, PDGF, and FGF-2. Dermal fibroblasts were cultured on native collagen with increasing concentrations of meglumine for 24 h and transcript levels were measured by quantitative real-time PCR. Fibroblasts showed a dose-dependent increase in the expression of COL1A1, α-SMA, TGF-β1, PDGF, and FGF-2 when cultured with meglumine (Fig. 49, p<0.05

and p<0.001). These results suggest that meglumine increases the basal transcript levels of critical wound healing components.

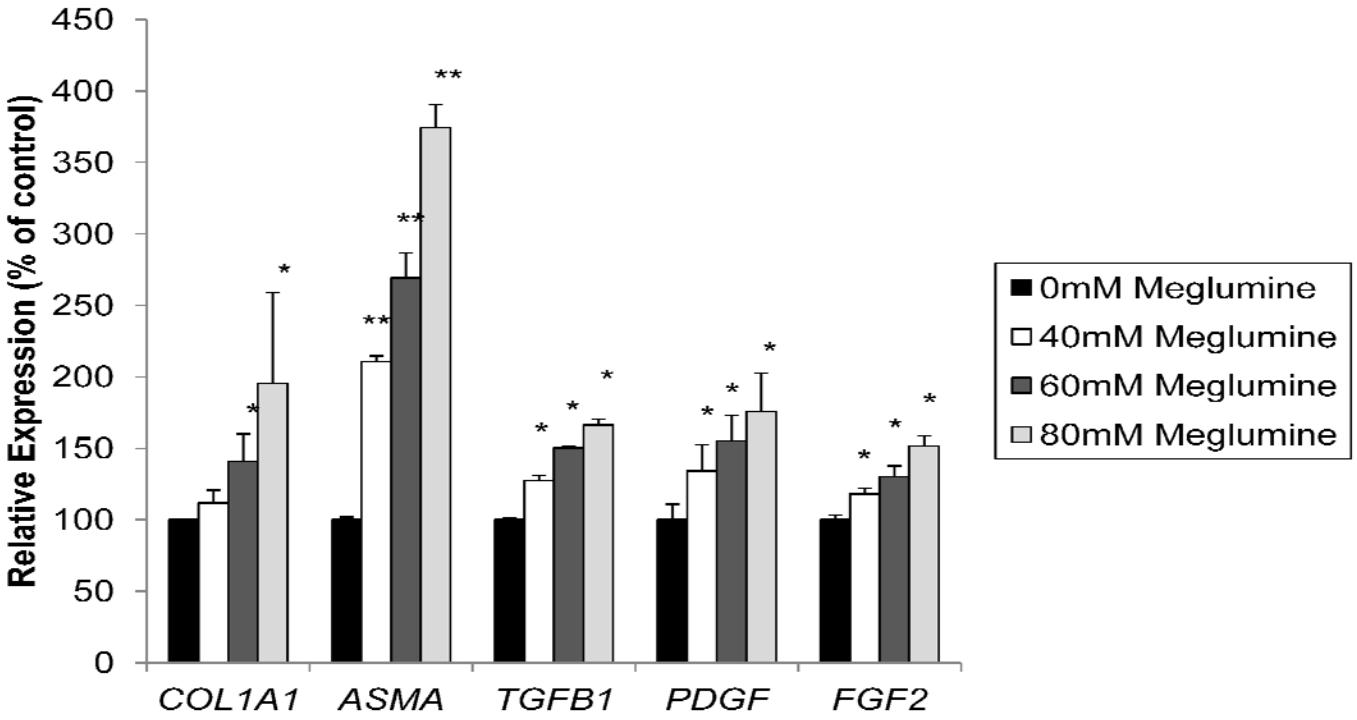

Figure 49. Effect of meglumine on the expression of wound healing components. 70% confluent fibroblasts were cultured on native collagen in the presence of 40 mM meglumine for 24 h. RNA was extracted and quantitative real-time PCR was performed for the following transcripts: COL1A1, α-SMA, TGF-β1, PDGF, and FGF-2. All transcript levels were normalized to β-actin. All comparisons were made against native collagen. Data are mean + SD (n=3), ****P < 0.001, *P < 0.05.**

Increased fibroblast migration after treatment with meglumine is dependent on p38 MAPK and ERK1/2. Fibroblasts control their migration into the wound site by activating growth kinases including p38 MAPK and AKT (41;53-55). It has been shown previously that fibroblasts control migration on native collagen through p38 MAPK dependent upregulation of AKT (Fig. 41 and 42). Furthermore, fibroblasts explanted from diabetic ulcers show a reduced capacity to migrate into the wound site. Therefore, we investigated the role of meglumine in wound closure and determined which kinases were responsible for promoting this wound closure. Fibroblasts were cultured on native collagen until confluent and then manually scratched with a pipette tip to induce an *in vitro* wound. Meglumine was added and the wound closure rates were measured at 24 h and 48 h. Meglumine had completely closed the wound (100% $\pm$ 1.4% by 48 h) compared to the 95% $\pm$ 1.4% wound closure in fibroblasts cultured on native collagen (Fig. 50, p<0.05). Inhibition of p38 MAPK with the inhibitor SB202190 in fibroblasts treated with meglumine reduced the wound closure rate to 70% $\pm$ 2.2% at 48 h, and this was comparable to that seen in fibroblasts cultured on native collagen and treated with SB202190 (p<0.005). Furthermore, inhibition of the pro-survival kinase AKT with the inhibitor LY294002 did not alter the wound closure rates after treatment with meglumine compared to fibroblasts treated with meglumine alone. This observation is in contrast to fibroblasts cultured on native collagen, where inhibition of AKT reduced the wound closure rates to 64% $\pm$ 2.8% at 48 h (p<0.005).

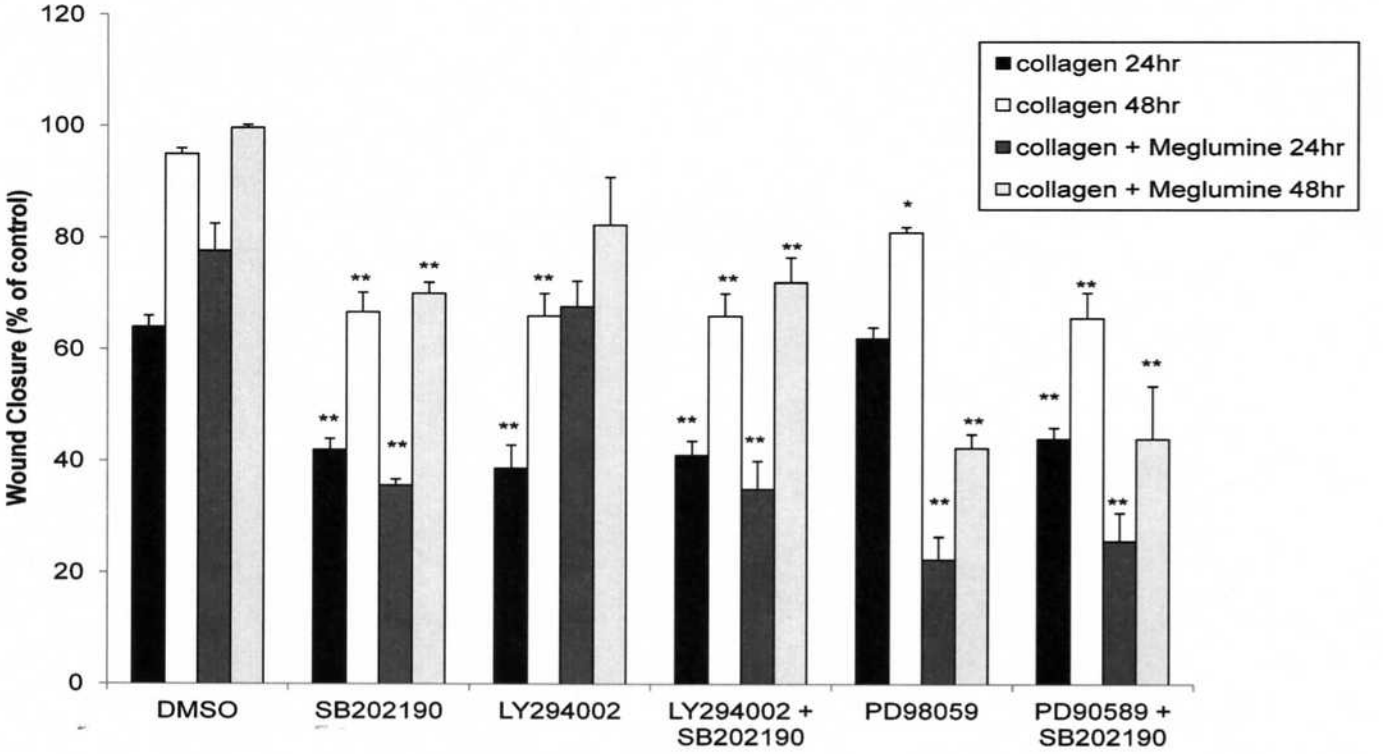

Figure 50. Wound closure rate after inhibition of p38 MAPK in fibroblasts treated with meglumine. Confluent fibroblasts were pretreated with the inhibitors; growth factor inhibitor suramin, p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, or the ERK1/2 inhibitor PD98059 for 1 h and cultured on native collagen. Fibroblasts were scratched manually with a pipette tip, cultured with 1% FBS DMEM supplemented with 40 mM meglumine, and the scratch wound was allowed to close. Cell migration into the wound was monitored at 24 h and 48 h by bright field visualization on an epi-fluorescence microscope. The distance across the wound margin was measured at 10 different points using Spot software and plotted as percentage of wound closure when compared to initial scratch at 0hr. Comparisons are performed within each time point and compared to that of collagen. Data are mean + SD (n=3), **$P < 0.005$, *$P < 0.05$.

These results suggest that AKT does not play a role in mediating meglumine induced fibroblast migration. Therefore, we investigated ERK1/2, which has been shown to play an important role in regulating fibroblast migration. Inhibition of ERK1/2 with the inhibitor PD98059 in fibroblasts treated with meglumine reduced the wound closure rate to 42% $\pm$ 3.0% at 48 h ($p<0.001$). Inhibition of both ERK1/2 and p38 MAPK did not further decrease the wound closure rates at 48 h (Fig. 47). These results suggest that p38 MAPK and ERK1/2 regulate meglumine induced fibroblast migration.

Increased proliferation after treatment with meglumine is dependent on p38 MAPK and ERK1/2. It has been shown that meglumine can promote growth factor expression which is involved in cell proliferation. Therefore, we investigated if meglumine can increase cell proliferation and which kinases are activated during cell proliferation. Inhibition of p38 MAPK reduced cell proliferation of fibroblast cultured on native collagen for up to 48 h (Fig. 51, $p<0.05$). In contrast, inhibition of AKT did not alter the rate of proliferation as fibroblasts treated with meglumine continued to proliferate up to 48 h. Inhibition of ERK1/2 reduced the rate of proliferation in fibroblasts treated with meglumine and the combined inhibition of p38 MAPK and ERK1/2 further enhanced this decline in proliferation (Fig. 51, $p<0.05$). These results suggest that meglumine mediates cell proliferation through activation of p38 MAPK and ERK1/2.

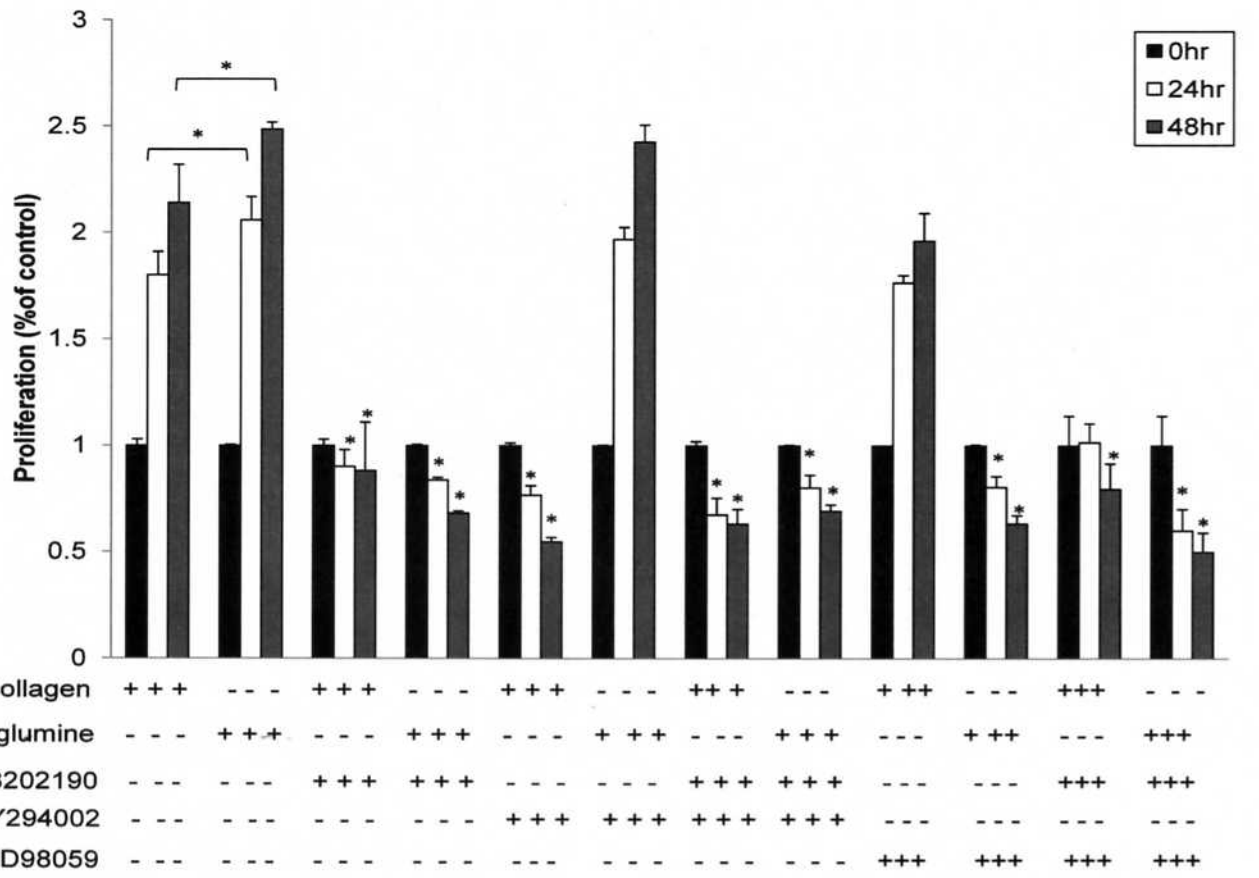

Figure 51. Proliferation rate after inhibition of p38 MAPK in fibroblasts treated with meglumine. 4 X 10^3 cells/well were pretreated with the growth factor receptor inhibitor suramin, p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, or ERK1/2 inhibitor PD98059 for 1 h and then seeded in a 96-well plate containing either native collagen or native collagen treated with 40 mM meglumine for 0 h, 24 h, and 48 h. At each designated time point the level of proliferation was determined using the cell proliferation reagent WST-1. Quantification of each sample was determined by measuring the absorbance at 450 nm, with a reference wavelength of 690 nm. Comparisons were performed within each time point and compared to native collagen alone. Data are mean $\pm$ SD (n=3), ***P < 0.05.**

Meglumine regulates cell survival through activation of p38 MAPK and ERK1/2. Diabetic wounds contain increased levels of apoptotic fibroblasts, which delays wound healing (27). Moreover, treatment of fibroblasts cultured on 3DG-collagen with meglumine reduced the levels of caspase-3, which is an early marker of apoptosis (Fig. 13). This suggests that meglumine plays a role in mediating cell survival. Because p38 MAPK and ERK1/2 in fibroblasts treated with meglumine promoted fibroblast migration and proliferation, we investigated the role of these kinases in mediating meglumine-induced cell survival. Fibroblasts pretreated with the p38 MAPK inhibitor SB202190 and treated with meglumine upregulated the level of caspase-3 activity to 149% $\pm$ 5.5% (Fig. 50, $p<0.001$). Inhibition of AKT with LY294002 did not alter the levels of caspase-3 suggesting that AKT is not responsible for mediating cell survival after treatment with meglumine. Inhibition of ERK1/2 with PD98059 upregulated the level of caspase-3 activity to 225% $\pm$ 6.0% ($p<0.0005$). Inhibition of both ERK1/2 and p38 MAPK did not induce a significant increase in the level of caspase-3 activity suggesting that both ERK1/2 and p38 MAPK play a role in mediating cell survival in fibroblasts treated with meglumine (Fig. 52).

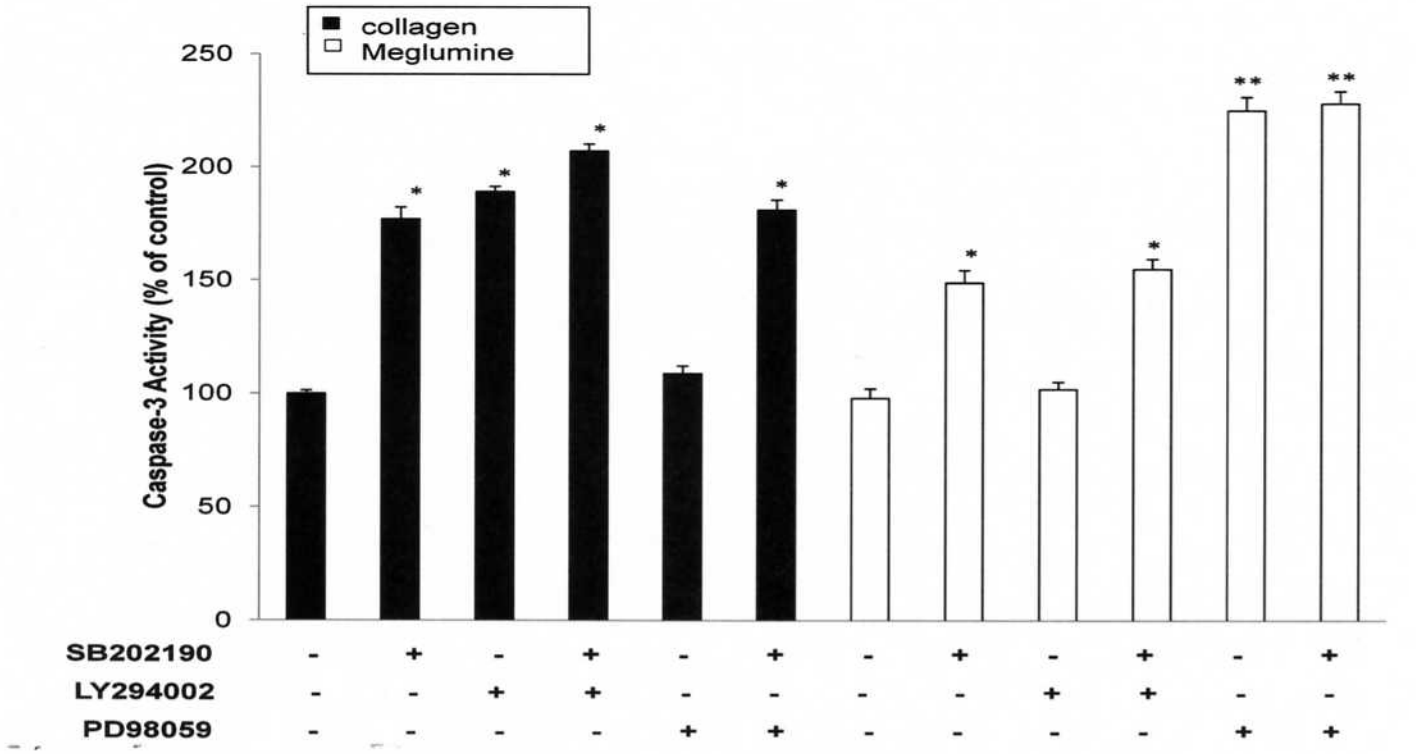

Figure 52. Measurement of caspase-3 activation after pretreatment with kinase inhibitors in fibroblasts treated with meglumine. Fibroblasts were pretreated with the inhibitors; p38 MAPK inhibitor SB202190, AKT inhibitor LY294002, or ERK1/2 inhibitor PD98059 for 1 h and then cultured on native collagen or treated with meglumine for 24 h. Whole cell lysates were assayed for caspase-3 activity according to the protocol from Caspase-3 Colorimetric Correlate Assay. All samples were performed in triplicate and normalized to the control samples. All comparisons are made against native collagen alone. Data are mean ± SD (n=3), **$P < 0.0005$, *$P < 0.001$.

Inhibition of p38 MAPK does not alter the phosphorylation of ERK1/2 in fibroblasts treated with meglumine. We show that phosphorylation of ERK1/2 is dependent on the activation of p38 MAPK in fibroblasts cultured on native collagen (Fig. 39). Since both p38 MAPK and ERK1/2 mediated migration, proliferation, and cell survival of fibroblasts treated with meglumine, we sought to understand better the cross-talk between these kinases. Fibroblasts were pretreated with the p38 MAPK inhibitor SB202190 and cultured on native collagen and treated with or without meglumine for 24 h. Western blot analysis revealed that in contrast to fibroblasts cultured on native collagen alone, fibroblasts treated with meglumine did not alter the phosphorylation of ERK1/2 after inhibition of p38 MAPK (Fig. 53). These results suggest that p38 MAPK and ERK1/2 are independently activated by meglumine to promote migration, proliferation, and cell survival.

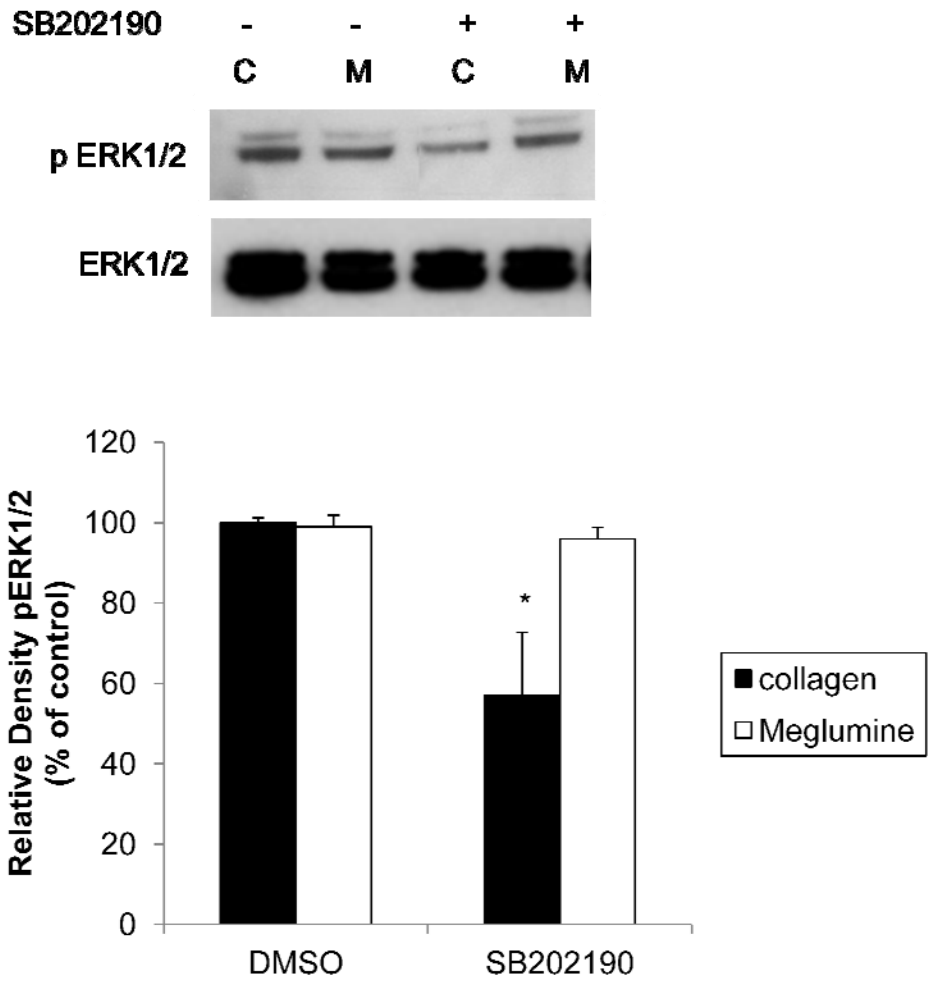

Figure 53. ERK1/2 phosphorylation after inhibition of p38 MAPK in fibroblasts treated with meglumine. Fibroblasts were pretreated with SB202190, or vehicle DMSO for 1 h and cultured on native collagen (C) or treated with meglumine (M) for 24 h. Expression of phopsho-ERK1/2 was analyzed by Western blot using whole cell lysates. Total ERK1/2 served as the loading control. The bars correspond to the densitometric values of phospho-ERK1/2 after normalization for total ERK1/2. All comparisons are made against native collagen treated with DMSO. Data are mean $\pm$ SD (n=3), ***P < 0.01.**

Meglumine abrogates the 3DG-collagen dependent downregulation of growth factors. Our previous studies have shown that fibroblasts cultured on 3DG-collagen have reduced levels of growth factor transcripts (Fig. 44). Since meglumine has been shown to upregulate growth factor expression at the level of transcription (Fig. 49), we investigated the role of meglumine in growth factor expression in fibroblasts cultured on 3DG-collagen. Fibroblasts were cultured on 3DG-collagen and treated with meglumine for 24 h and quantitative real-time PCR was performed. Fibroblasts treated with meglumine and cultured on 3DG-collagen abrogated the downregulation of these growth factors and the expression levels of TGF-β1, PDGF, and FGF-2 were similar to that seen in fibroblasts cultured on native collagen (Fig. 54; $p<0.05$). These results suggest that meglumine restores the expression of growth factors in fibroblasts cultured on 3DG-collagen.

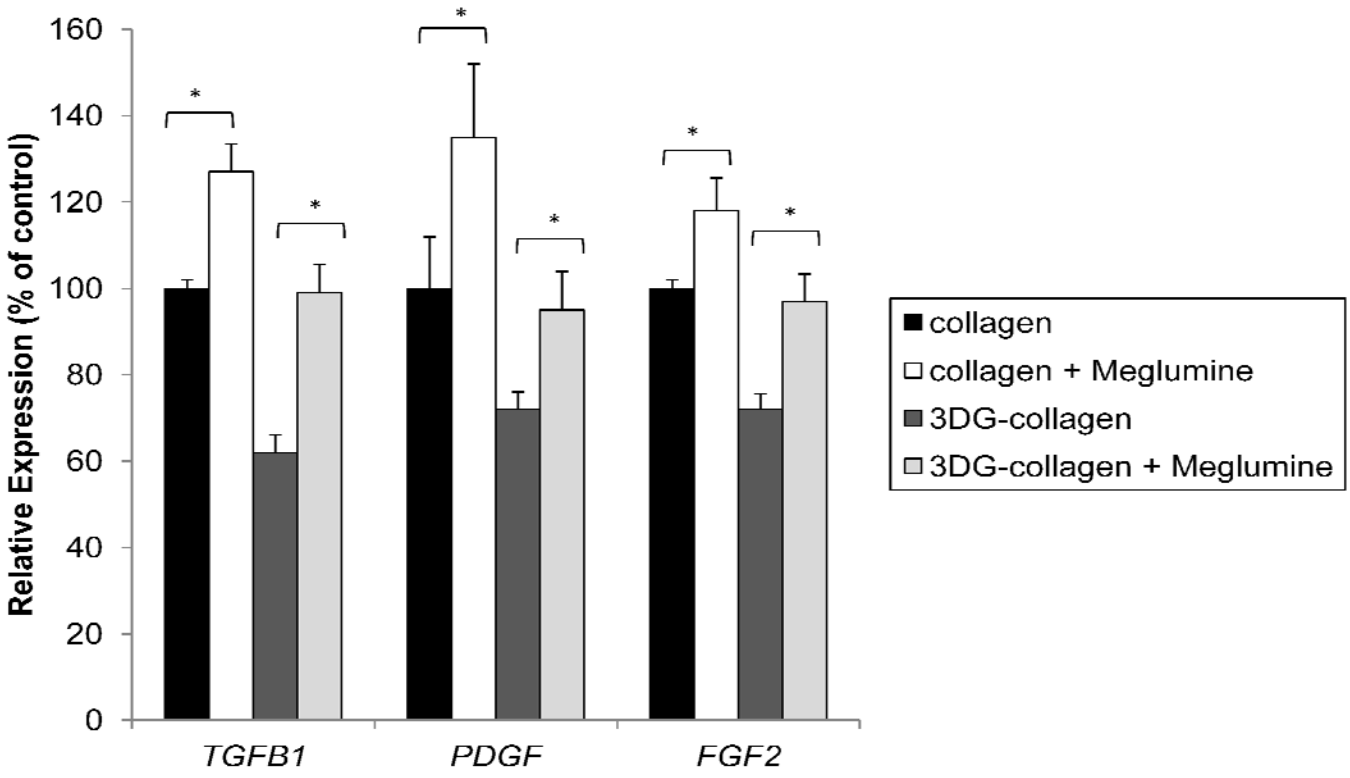

Figure 54. Effect of meglumine on 3DG-collagen dependent regulation of growth factor expression. 70% confluent fibroblasts were cultured on native collagen or 3DG-collagen in the presence and/or absence of 40 mM meglumine for 24 h. RNA was extracted and quantitative real-time PCR was performed for the following transcripts: TGF-β1, PDGF, and FGF-2. All transcript levels were normalized to β-actin. All comparisons were made against native collagen. Data are mean $\pm$ SD (n=3), ***P < 0.05.**

Meglumine prevents increased fibroblast adhesion to 3DG-collagen. Fibroblasts have been shown to adhere stronger to 3DG-collagen than native collagen (Fig. 17) and this adhesion was dependent on the downregulation of growth factors (Fig.44). Since meglumine could reverse the effects of 3DG-collagen-induced downregulation of growth factors (Fig. 54), we investigated the role of meglumine on cell adhesion. Fibroblasts treated with meglumine for 3 h reduced their adhesion to 3DG-collagen to 97% $\pm$ 4.0%, which was comparable to fibroblast adhesion to native collagen. The reduced adhesion by meglumine continued for 24 h where the relative adhesion to 3DG-collagen was 10% $\pm$ 6.6% of that seen in fibroblasts adhering to native collagen at 24 h (Fig. 55; $p<0.01$).

Meglumine abrogates the increased expression of α1β1 integrin in fibroblasts cultured on 3DG-collagen. α1β1 integrin are the key collagen receptors in dermal fibroblasts responsible for the increased binding to 3DG-collagen (Fig. 45). This increase in transcription of α1β1 integrin causes a shift in the dynamics of adhesion between the fibroblast and 3DG-collagen, which results in reduced mobility of the fibroblast (Fig. 41). Since meglumine abrogated the strong adhesion between fibroblasts and 3DG-collagen (Fig. 55), we investigated the role of meglumine in altering the expression of α1β1 integrin in fibroblasts cultured on 3DG-collagen. Quantitative real-time PCR revealed a decrease in the expression of both α1 and β1 integrin in fibroblasts treated with meglumine in the presence of 3DG-collagen (Fig. 56; $p<0.001$). This reduction in transcript levels was comparable to that observed in fibroblasts cultured on native collagen. Therefore, these results suggest that meglumine may reduce adhesion through reduction in the transcript levels of α1β1.

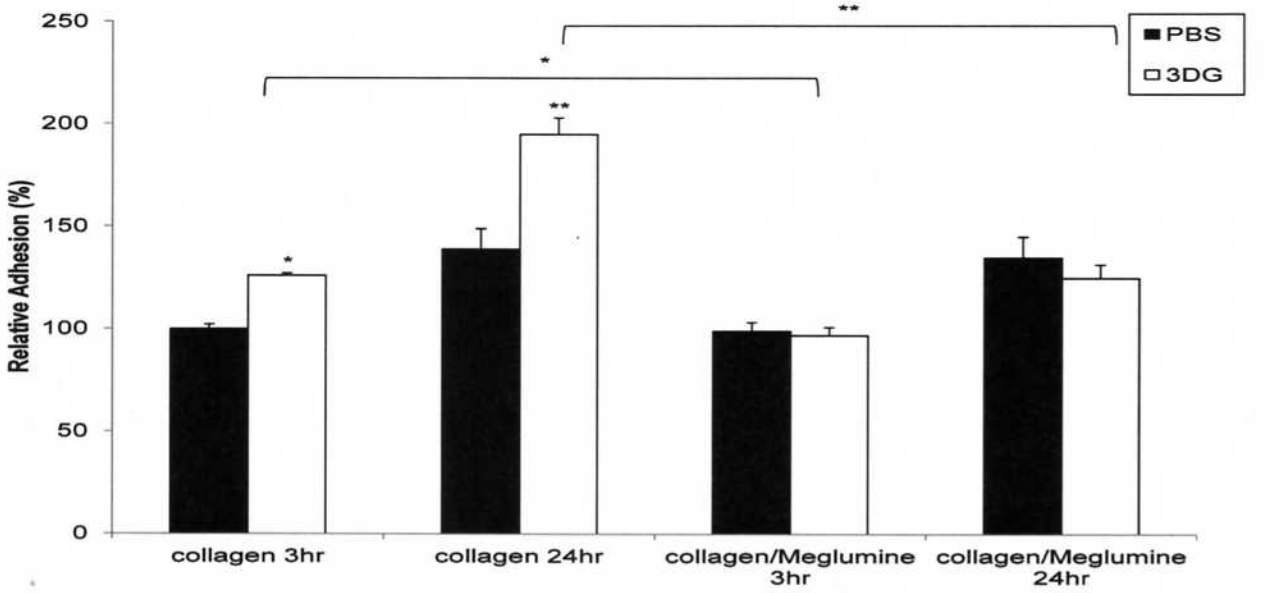

Figure 55. Reduced adhesion to 3DG-collagen after treatment with meglumine. Fibroblasts were seeded on native collagen and 3DG-collagen treated with or without meglumine, and allowed to attach for 3h and 24h. Fibroblast adhesive strength was measured by counting the cells remaining after a jet wash assay. Cell numbers from 10 random images were counted and averaged, and presented as percentage of adhesion. Comparisons were performed against native collagen at 3h and 24h, respectively. Data are mean ± SD (n=3), ****P < 0.01, *P < 0.03.**

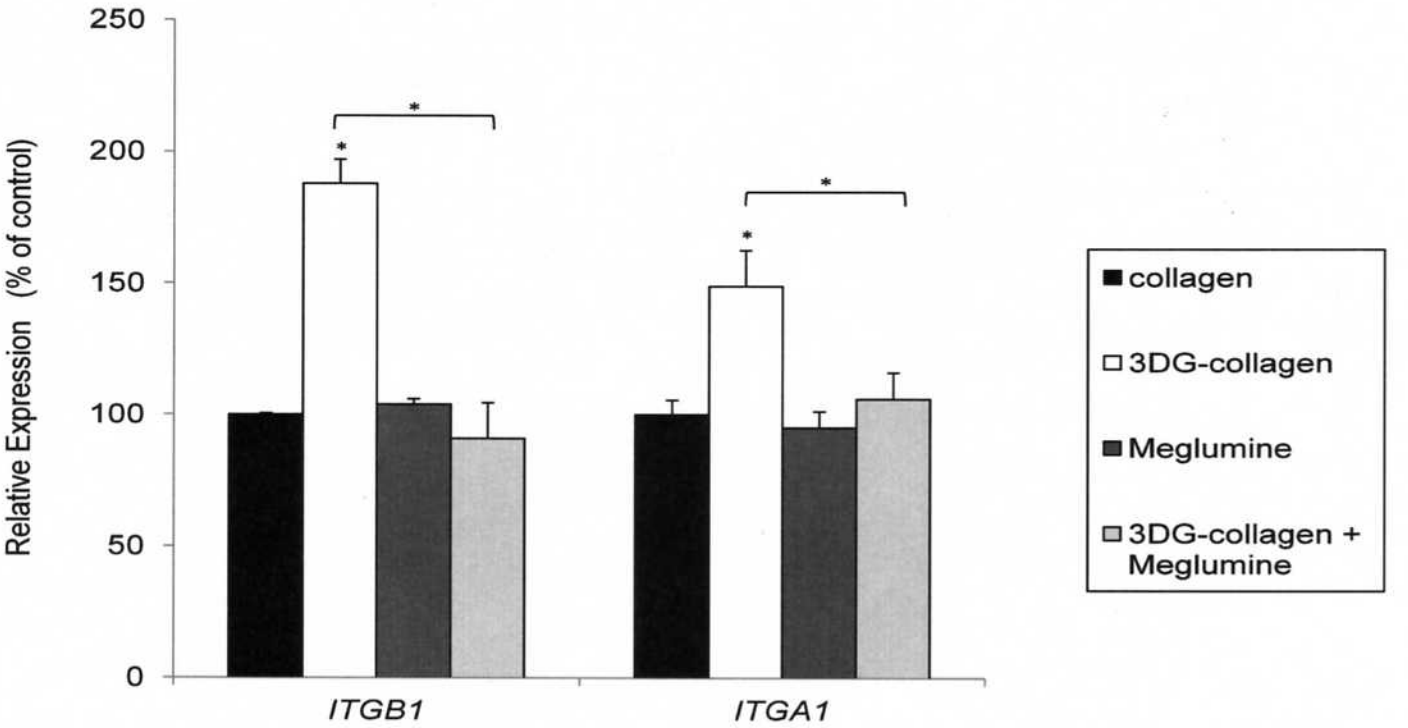

Figure 56. Reduced expression of α1β1 integrin after treatment with meglumine in fibroblasts cultured on 3DG-collagen. 70% confluent fibroblasts were cultured on native collagen or 3DG-collagen in the presence and/or absence of 40 mM meglumine for 24 h. RNA was extracted and quantitative real-time PCR was performed for the following transcripts: α1 ITG and β1 ITG. All transcript levels were normalized to β-actin. All comparisons were made against native collagen unless otherwise noted. Data are mean ± SD (n=3), ***P < 0.001**.

Meglumine restores migration in fibroblasts cultured on 3DG-collagen. We demonstrated a reduction in fibroblast migration when cultured on 3DG-collagen (Fig. 41). Since meglumine has been shown to reverse the increased adhesion and reduced gr owth factor expression induced by 3DG-collagen (Figs 54-55), we investigated the role of meglumine in fibroblast migration into a wound coated with 3DG-collagen. Utilizing an *in vitro* scratch assay, fibroblasts were cultured on 3DG-collagen until confluent and a scratch was made across the monolayer of cells. After the scratch was m ade, 3DG-collagen was then reintroduced to the scratch for an additional 2 h. Meglum ine was then added to the remaining fibroblasts and the wound closure rate was measured over 48 h. The addition of meglumine to fibroblasts cultured on 3DG-collagen restored migration as 95% of the wound was closed by 48 h. (Fig. 57; $p<0.02$). These results suggest that m eglumine is able to restore the reduced migration seen in fibroblasts cultured on 3DG-collagen.

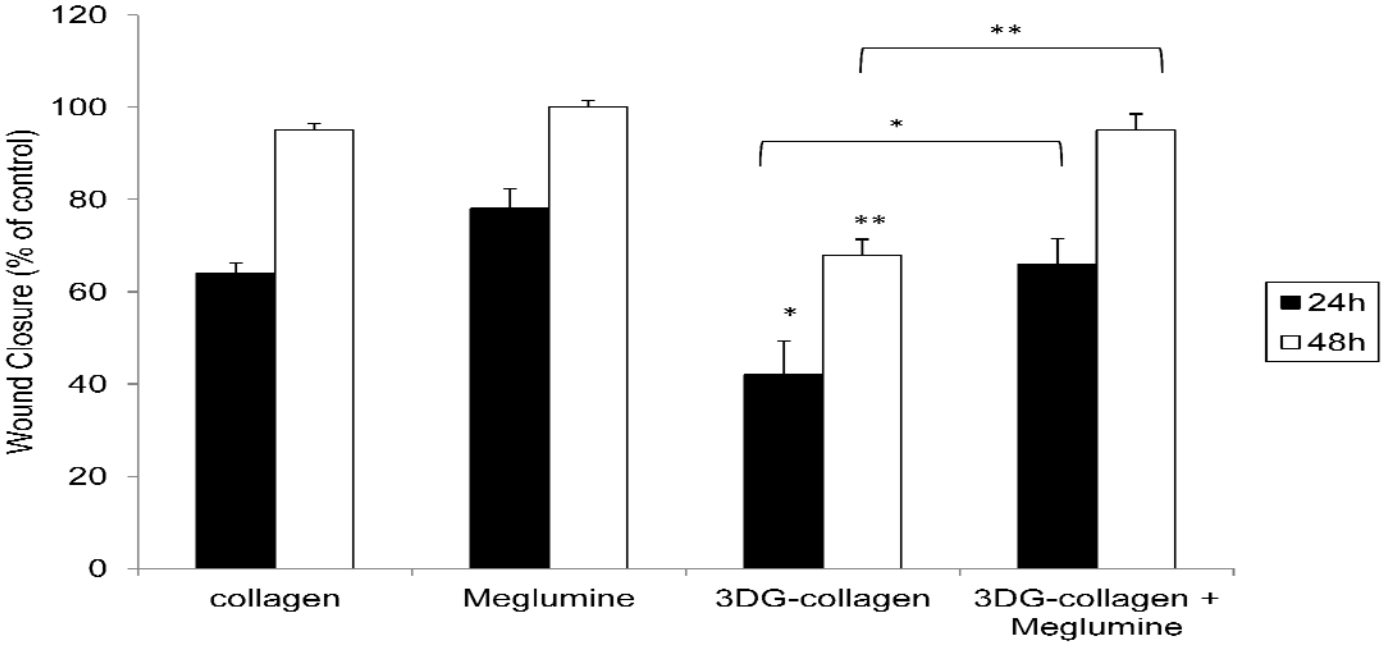

Figure 57. Wound closure rate after treatment with meglumine in fibroblasts cultured on 3DG-collagen. Confluent fibroblasts were cultured on native collagen or 3DG-collagen. Fibroblasts were scratched manually with a pipette tip, cultured with 1% FBS DMEM supplemented with 40 mM meglumine, and the scratch wound was allowed to close. Cell migration into the wound was monitored at 24 h and 48 h by bright field visualization on an epi-fluorescence microscope. The distance across the wound margin was measured at 10 different points using Spot software and plotted as percentage of wound closure when compared to initial scratch at 0hr. Comparisons are performed within each time point and compared to that of collagen. Data are mean + SD (n=3),
****P < 0.02, *P < 0.05.**

Meglumine abrogates the downregulation of collagen transcription by 3DG-collagen. Wound healing is characterized by fibroblast m igration, proliferation, and production of ECM components such as type I collagen. 3DG-collagen has been shown to negatively im pact the transcription of type I collagen (COL1A1)(45 and Fig. 7). Furtherm ore, meglumine has been shown to dose-dependently upregulate the tra nscription of COL1A1 (Fig. 49). Therefore, we i nvestigated if meglumine had the ab ility to restore COL1A1 expression in fibroblasts cultured on 3DG- collagen. Fibroblasts cultured on 3DG- collagen and treated with m eglumine increased their expression of COL1A1 to that seen in fibroblasts cultured on native collagen alone ($p<0.05$; Fig. 58). These results suggest that meglumine can reverse the downregulation of collagen expression in fibroblasts cultured on 3DG-collagen.

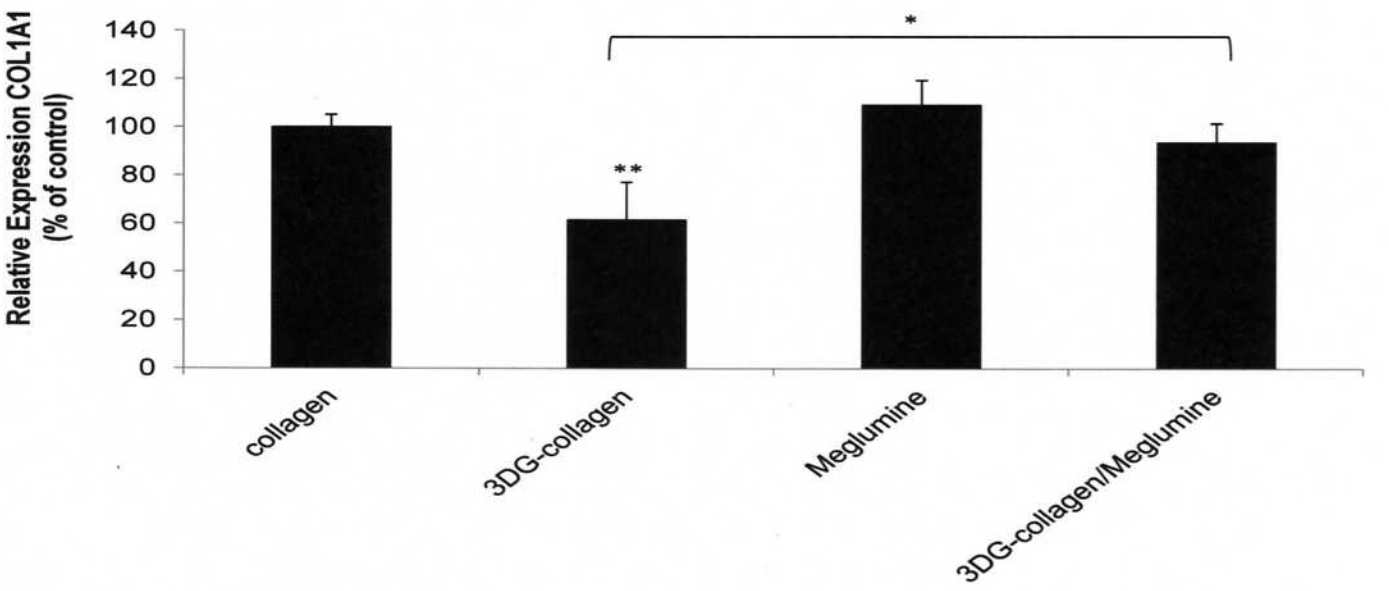

Figure 58. Expression of COL1A1 after treatment with meglumine. 70% fibroblasts were cultured on native collagen or 3DG-collagen in the presence or absence of meglumine for 24 h. RNA was extracted and quantitative real-time PCR was performed for the COL1A1 transcript. Transcript levels were normalized to β-actin. All comparisons were made against native collagen unless otherwise noted. Data are mean ± SD (n=3), **$^{**}P < 0.01$, $^{*}P < 0.05$.

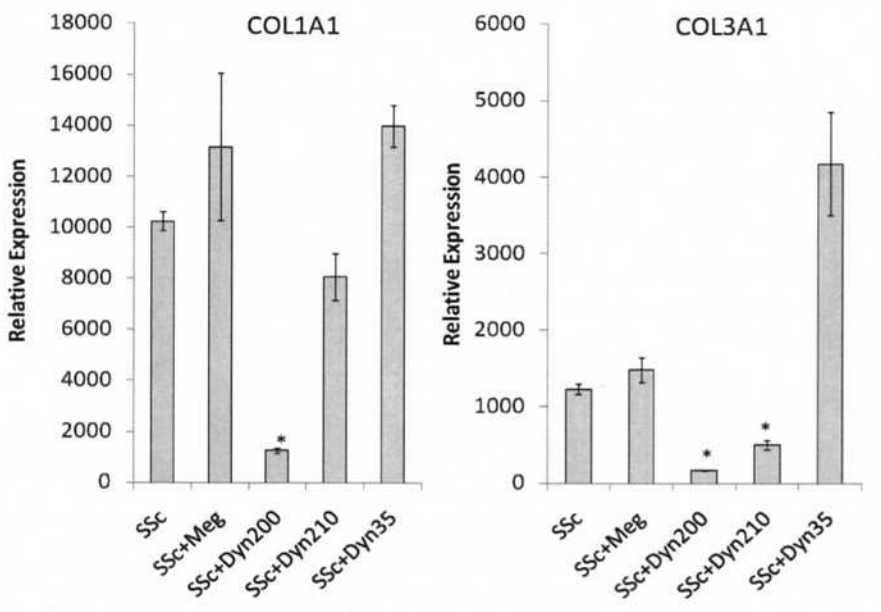

Figure 59. Analogs of meglumine alter collagen expression. SSc fibroblasts were treated with 40 mM meglumine (Meg), 1 mM Dyn200, 1 mM Dyn210, and 0.4 mM Dyn35 for 24 h. RNA was harvested and measured for COL1A1 and COL3A1 transcripts **$^{*}p<0.05$.

Analogs of Meglumine. We investigated analogs of meglumine as we found that meglumine could be used therapeutically for wound healing as it increased collagen and cell migration, however for it to be effective 40mM had to be used. Therefore, we made additional analogs of meglumine (Dyn35, Dyn200, Dyn210) and tested these to determine their effect on SSc fibroblasts. As expected, meglumine (Meg) increase collagen expression (Fig. 59). However two analogs of meglumine, Dyn200 and Dyn210, decreased collagen expression in SSc fibroblasts; whereas we found Dyn35 increased collagen expression (Fig. 59). This finding implicates that we may have identified at least one possible therapeutic (Dyn200) that could be used to lower collagen expression in SSc fibroblasts. When we studied TGF-β1 transcripts, we found that this was also decreased although not significant (Fig. 60). We will be further pursuing Dyn200 as a candidate drug for the treatment of scleroderma in a subsequent SBIR application to NIH.

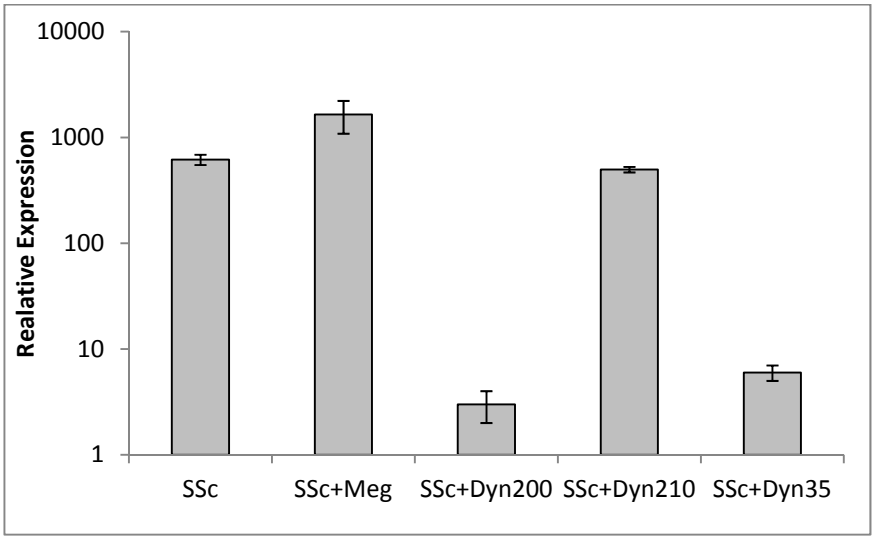

Figure 60. Analogs of meglumine alter TGF-β1 expression. SSc fibroblasts were treated with 40 mM meglumine (Meg), 1 mM Dyn200, 1 mM Dyn210, and 0.4 mM Dyn35 for 24 h. RNA was harvested and measured for COL1A1 and COL3A1 transcripts.

Key Research Accomplishments

1. Collagen expression declines with chronological age (Fig. 1)
2. Fibroblasts from older individuals are more sensitive to 3DG-collagen (Fig. 2)
3. The number of cell doublings sensitizes fibroblasts to 3DG-collagen (Fig. 3)
4. Unaffected fibroblasts and affected fibroblasts from the same patient did not always have the level of collagen expression expected and therefore chosen resources needed to be tested (Fig. 4)
5. The addition of 3DG reduced collagen expression in SSc fibroblasts (Fig. 5)
6. The metabolism of fructoselysine to released 3DG intracellularly also decreased collagen (Fig. 5)
7. 3DG from fructoselysine had a greater effect at reducing collagen than commercially available 3DG (Fig. 5)
8. Advanced glycation end products do not have the same effect on modifying collagen. For example MG increases the apparent molecular weight of collagen whereas 3DG does not (Fig. 6).
9. The decrease in collagen expression with morpholinofructose (also able to be metabolized to 3DG) translates to a concordant decrease in total protein secreted by fibroblasts.
10. 3DG titration induces a greater decrease in COL1A1 and COL3A1 transcripts (Fig. 7)
11. 3DG titration induces a greater decrease in COL1A1 and COL3A1 protein (Fig. 7)
12. MG titration induces a greater increase in COL1A1 and COL3A1 transcripts (Fig. 7)
13. MG titration induces a greater increase in COL1A1 and COL3A1 protein (Fig. 7)
14. 3DG decreases TGF-β1 transcripts and protein (Fig. 8)
15. 3DG decreases β1 integrin transcripts and protein (Fig. 8)
16. MG increases TGF-β1 transcripts and protein (Fig. 8)
17. MG increases β1 integrin transcripts and protein (Fig. 8)
18. Proliferation of fibroblasts is reduced with 3DG and MG (Fig. 9A)
19. Dyn15/meglumine induces the proliferation of fibroblasts (Fig. 9B)
20. 3DG-collagen reduces the phosphorylation of ERK1/2 and MEK1/2 in normal and SSc fibroblasts (Fig. 11)
21. Cytoplasmic distribution of phosphorylated ERK1/2 and MEK1/2 is lower in fibroblasts cultured on 3DG-collagen (Fig. 12)
22. Caspase-3 is induced with 3DG and morpholinofructose (MF) (Fig. 13)
23. caspase-3 induction by 3DG and MF can be abolished by Dyn15/meglumine (Fig. 13)
24. The distal promoter of collagen is activated in fibroblasts cultured on native collagen (Fig. 14)

25. Binding of protein to the distal promoter is abolished when fibroblasts are cultured on 3DG-collagen (Fig. 14)
26. Transcription factors Sp1, c-fos, and c-myc are reduced by 3DG-collagen (Fig. 15)
27. Smad7 expression is increased by 3DG-collagen (Fig. 16)
28. Fibroblasts adhere more strongly to 3DG-collagen (Fig. 17)
29. In fibroblasts cultured on 3DG-collagen, paxillin and focal adhesion kinase locate to the perinuclear region of the cell (Fig. 18)
30. Focal adhesion kinase is decreased in fibroblasts grown on 3DG-collagen but not paxillin (Fig. 19)
31. 3DG increases α1 integrin transcripts and protein (Figs. 20-21)
32. 3DG increases α5 integrin transcripts and protein (Figs. 20-21)
33. 3DG increases αv integrin transcripts and protein (Figs. 20-21)
34. 3DG increases β3 integrin transcripts and protein (Figs. 20-21)
35. 3DG decreases α2 integrin transcripts and protein (Figs. 20-21)
36. 3DG decreases β1 integrin transcripts and protein (Figs. 20-21)
37. α1β1 integrin is required for the fibroblasts to bind to 3DG-collagen (Fig. 22)
38. 3DG-collagen increases the protein levels of GADD153 (Figs. 23-24)
39. Meglumine, salubrinal, and aminoguanidine prevent the increase of GADD153 by 3DG-collagen (Figs. 23-24)
40. 3DG-collagen induces intracellular ROS (Fig. 25)
41. Meglumine and ascorbic acid abolish ROS production by 3DG-collagen (Fig. 25)
42. 3DG-collagen induces the expression of NAD(P)H oxidase 4 transcripts and protein (Figs. 26-27)
43. Meglumine abolishes the induction of Nox 4transcripts and protein (Figs. 26-27)
44. Nox4 expression is mediated by ROS (Fig. 28)
45. Phosphorylation of p38 MAPK is dependent on 3DG-collagen induced ROS (Fig. 29)
46. Inhibition of ROS and p38 MAPK abrogates GADD153 expression (Fig. 30)
47. GADD153 expression is dependent on p38 MAPK (Fig. 31)
48. Inhibition of ROS and p38 MAPK abrogates caspase-3 activity (Fig. 32)
49. 3DG-collagen does not induce the expression of RAGE transcripts and protein (Fig2. 33-34)
50. MG-collagen does induce the expression of RAGE transcripts and protein (Fig2. 33-34)
51. Inhibition of RAGE does not alter the ER stress pathway in fibroblasts cultured on 3DG-collagen (Fig. 35)
52. Inhibition of RAGE signaling does alter the ER stress pathway in fibroblasts cultured on MG-collagen (Fig. 35)
53. 3DG-collagen activates the ER stress signaling cascade (GADD153, caspase-3, ROS) via α1β1 integrin (Fig. 36)
54. Phosphorylated p38 MAPK is a transient signal in fibroblasts cultured on 3DG-collagen (Fig. 37)
55. 3DG-collagen decreases H-ras but upregulates the phosphorylation of ASK1 (Fig. 38)
56. Suramin which inhibitors growth factors, reverses the effects of 3DG-collagen on H-ras and ASK1 protein levels (Fig. 38)
57. Inhibition of p38 MAPK with SB202190 induces phosphorylation of ERK1/2 in fibroblasts cultured on 3DG-collagen (Fig. 39)
58. Inhibition of p38 MAPK with SB202190 induces phosphorylation of Akt in fibroblasts cultured on 3DG-collagen (Fig. 39)
59. Inhibition of p38 MAPK with SB202190 decreases phosphorylation of ERK1/2 in fibroblasts cultured on native collagen (Fig. 39)
60. Inhibition of p38 MAPK with SB202190 decreases phosphorylation of Akt in fibroblasts cultured on native collagen (Fig. 39)
61. p38 MAPK regulates the migration of fibroblasts when cultured on 3DG-collagen or native collagen (Fig. 40)
62. The inhibition of fibroblast migration on 3DG-collagen was not dependent on ERK1/2, however it was dependent on Akt (Fig. 40)
63. Inhibition of p38 MAPK delays filopodia extension by fibroblasts (Fig. 41)

64. H-ras dependent activation is required for filopodia extension by fibroblasts whereas ASK1 inhibits filopodia extension (Fig. 41)
65. Fibroblasts proliferation is dependent on p38 MAPK activation of Akt (Fig. 42)
66. The requirement of α1β1 integrin by 3DG-collagen is dependent on growth factor signaling and H-ras expression (Fig. 43)
67. Blockade of α1β1 abrogates 3DG-collagen induced downregulation of growth factors (Fig. 44)
68. Blockade of α1β1 abrogates 3DG-collagen induced downregulation of fibroblast migration (Fig. 45)
69. Akt phosphorylation is required for regulation of p38 MAPK induced caspase-3 activity in fibroblasts cultured on native collagen (Fig. 46)
70. In fibroblasts cultured on 3DG-collagen, p38 MAPK reduces the phosphorylation of Akt and this leads to increased caspase-3 activity (Fig. 46)
71. Type I collagen is inversely regulated by p38 MAPK in fibroblasts cultured on native collagen and 3DG-collagen (Fig. 47)
72. COL1A1 and COL3A1 expression is decreased by inhibiting p38 MAPK and Akt signaling (Fig. 48)
73. Meglumine induces COL1A1, α-SMA, TGF-β1, PDGF, and FGF2 (Fig. 49)
74. Increased fibroblast migration by meglumine occurs via p38 MAPK and ERK1/2 (Fig. 50)
75. Increased cell proliferation by meglumine occurs via p38 MAPK and ERK1/2 (Fig. 51)
76. Meglumine inhibits caspase-3 activity and this occurs through p38 MAPK and ERK1/2 (Fig. 52)
77. Meglumine induces the phosphorylation of ERK1/2 (Fig. 53)
78. Meglumine abrogates the decreased expression of fibroblasts growth factors when cultured on 3DG-collagen (Fig 54.)
79. Meglumine increases fibroblast adhesion to 3DG-collagen (Fig. 55)
80. Meglumine abrogates the increased expression of α1β1 integrin when fibroblasts are cultured on 3DG-collagen (Fig. 56)
81. Meglumine increases the wound closure rate of fibroblasts cultured on 3DG-collagen (Fig. 57)
82. Meglumine induces collagen even in the presence of 3DG-collagen (Fig 58)
83. SSc fibroblasts have increased Hic-5 levels and the levels are more pronounced in affected/involved fibroblasts (Fig. 60)
84. Hic-5 expression is induced by 3DG and abolished by meglumine (Fig. 61)
85. Analogs of meglumine alter collagen expression (Fig. 62)
86. Dyn200 and Dyn210 are potential candidate drugs for SSc fibrosis as they reduce collagen expression (Fig. 62)
87. Dyn35 is a potential candidate for wound healing and collagen induction (Fig. 62)
88. Analogs of meglumine also alter TGF-β1 expression (Fig. 63)

Reportable Outcomes

Publications

1. Loughlin DT, Artlett CM. 3-Deoxyglucosone-Collagen Alters Human Dermal Fibroblast Migration and Adhesion: Implications for Impaired Wound Healing in Patients with Diabetes. Wound Repair and Regeneration **17**:739-749, 2009.

2. Sassi-Gaha S, Loughlin DT, Kappler F, Schwartz ML, Su B, Tobia AM, Artlett CM. Two dicarbonyl compounds, 3-deoxyglucosone and methylglyoxal, differentially modulate dermal fibroblasts. Matrix Biology **29**:127-134, 2010.

3. Loughlin DT, Artlett CM. Precursor of Advanced Glycation End Products 3-deoxyglucosone Mediates ER-stress-induced Caspase-3 Activation of Human Dermal Fibroblasts through NAD(P)H Oxidase 4. PLOS One **5**: e11093, 2010.

4. Loughlin DT, Artlett CM. Modification of collagen by 3-deoxyglucosone alters wound healing through differential regulation of p38 MAPK. PLOS One **6** :e18676, 2011.

Manuscripts in Preparation

1. Sassi-Gaha S, Holmgren AM, Feghali-Bostwick CA, Artlett CM. Decreased expression of extracellular matrix molecules in systemic sclerosis dermal fibroblasts utilizing the glucose metabolite, 3-deoxyglucosone.
2. Loughlin DT, Sassi-Gaha S, Tobia AM, Artlett CM. Meglumine Enhances Dermal Fibroblast Migration and Proliferation: A Potential Therapeutic for Diabetic Wounds.

Abstract Presentation at Meetings

1. Sassi-Gaha S. Artlett CM. Advanced glycation end products 3DG and MG, alter collagen expression by causing fibroblasts to respond differently. Discovery Day 2007.
2. Loughlin DT, Artlett CM. 3-Deoxyglucosone modifies long lived proteins in an age-dependent manner. Discovery Day 2007.
3. *Artlett CM, Sassi-Gaha S, Loughlin DT. Decreased Expression of Pertinent Extracellular Matrix Molecules in Systemic Sclerosis Utilizing a Glucose Metabolite that Modulates p44/p42 MAP Kinase pathway. American College of Rheumatology, October 2008.
4. Bakillah H, Sassi-Gaha S, Artlett CM. Dose Response of Meglumine on Fibroblast Collagen Expression over Time. Discovery Day 2008.
5. Holmgren AM, Sassi-Gaha S, Loughlin DT, Artlett CM. Older Individuals Express more Hic-5 and 3DG induced Hic-5 to Shuttle into the Nucleus. Discovery Day 2008.
6. Loughlin DT, Artlett CM. 3-Deoxyglucosone Modifies Fibroblast Focal Adhesions and Induces GADD153 Leading to Altered Wound Healing. Discovery Day 2008.
7. Sassi-Gaha S, Artlett CM. 3-Deoxyglucosone Alters Integrin Expression and Collagen Specific Transcription Factors in Scleroderma Fibroblasts. Discovery Day 2008.
8. Artlett CM, Loughlin DT, Feghali-Bostwick CA, Sassi-Gaha S. Decreased extracellular matrix molecules in systemic sclerosis fibroblasts utilizing the glucose metabolite 3-deoxyglucosone (3DG). Military Health Research Forum 2009.
9. Loughlin DT, Sassi-Gaha S, Artlett CM. 3-Deoxyglucosone-modified collagen induces apoptosis of dermal fibroblasts through activation of GADD153 via p38 MAPK. Platform presentation (winner), Discovery Day 2009.
10. Sassi-Gaha S, Loughlin DT, Feghali-Bostwick CA, Artlett CM. Decreased extracellular matrix molecules in systemic sclerosis fibroblasts utilizing the glucose metabolite 3-deoxyglucosone (3DG). Discovery Day 2009.

* This was a podium presentation at the American College of Rheumatology Conference in San Francisco, October 2008 in the Scleroderma Concurrent session. Six abstracts were presented in this concurrent session, selected from 137 accepted abstracts for scleroderma and out of a total of 2076 accepted abstracts for the entire meeting.

Lecture

Loughlin DT, Artlett CM. Role of 3-deoxyglucosone in impaired wound healing: building a mechanism. Presented to Microbiology and Immunology, Drexel University College of Medicine, April 2009.

Conclusion. Signaling pathways involved in 3DG-collagen effects on fibroblasts. Our studies have significantly expanded the current knowledge regarding fibroblast interactions with 3DG-collagen. These studies have provided the basis for a novel mechanism by which 3DG-collagen can negatively regulate fibroblast signaling to reduce proliferation, migration, survival, and ECM components. The mechanisms delineated by the present findings within this report provide a significant role for α1β1 integrin and growth factor receptors in regulating fibroblast signaling by 3DG-collagen. As presented in Fig. 61, 3DG-collagen may

interact with α1β1 integrin on the surface of fibroblasts causing a downregulation in the transcription of growth factors, PDGF, FGF-2, and TGF-β1 and their corresponding receptors. This reduced expression of growth factor receptors on the cell surface, would then reduce downstream signaling through H-ras and ERK1/2. H-ras is known to suppress integrin activation and so its downregulation by 3DG-collagen was shown to induce the transcription of α1β1 integrin. The overwhelming activation of α1β1 integrin in the absence of growth factor signaling may result in increased adhesion and increased ROS production through Nox4 upregulation. The increased adhesion would then result in the cell becoming static and incapable of extending its filopodia and migrating, resulting in reduced fibroblast migration along 3DG-collagen. Furthermore, the increased ROS production caused oxidative stress within the cell resulting in ER stress. Uncontrolled ER stress, as seen with 3DG-collagen, would then result in the induction of GADD153 via p38 MAPK and activate caspase-3 causing activation of the early apoptotic cascade. In addition to this, under times of oxidative stress p38 MAPK can be activated by ASK1 to reduce the phosphorylation of ERK1/2 and AKT further reducing the migration, proliferation, and survival of the fibroblast when cultured on 3DG-collagen. These events would then cause aberrant wound healing that is similar to that seen in diabetic wounds, a mechanism that could be employed in the fibrotic disease scleroderma. Additionally, the stress activated p38 MAPK would then further exacerbate reduced wound healing by decreasing the transcription and expression of type I collagen through downregulation of ERK1/2 and AKT. Identification of two novel collagen inhibitors has led to the pending submission of an SBIR grant application to NIH.

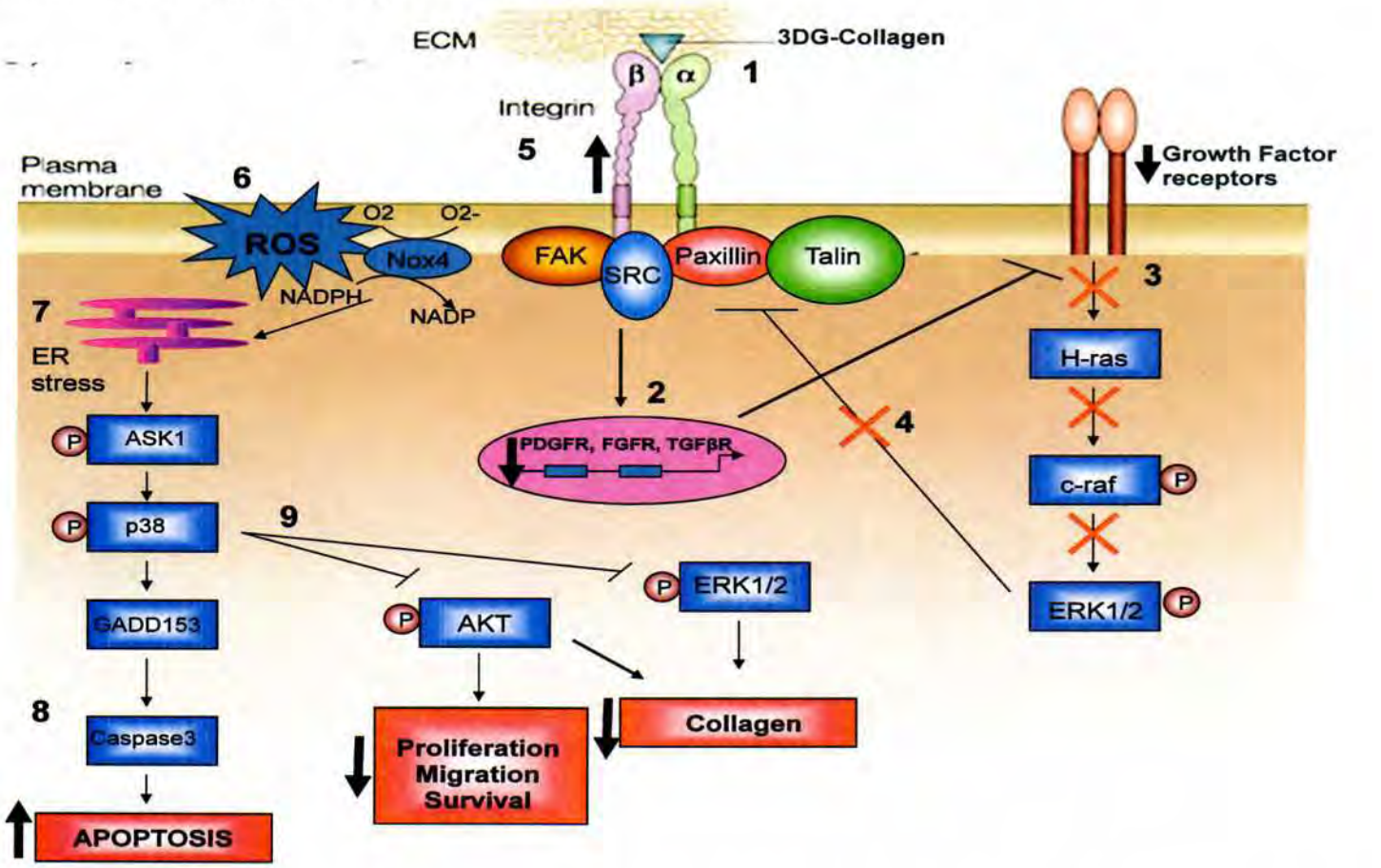

Figure 61. Proposed mechanism of fibroblast signaling when cultured on 3DG-collagen. (1) 3DG-collagen interacts with α1β1 integrin on the surface of the fibroblast causing downregulation in the transcription of growth factors and receptors (2). This then causes a downregulation in growth factor signaling via H-ras, (3) which results in the transcription of α1β1 integrin (4) and increased surface expression of α1β1 integrin (5) resulting in increased adhesion. This increased adhesion and reduced growth factor signaling induces activation of Nox4 and the production of ROS (6). Uncontrolled production of ROS leads to ER stress (7) and activation of ER stress induced apoptosis marker GADD153 causing caspase-3 activation (8). Furthermore, increased phosphorylation of p38 MAPK suppresses the phosphorylation of AKT and ERK1/2 reducing the overall migration, proliferation, and survival of the fibroblast as well as reducing the expression of type I collagen (9). Red X denotes the negative effect of 3DG-collagen on that pathway.

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